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Genetic-Diagnostic Survey

in Intellectually Disabled Individuals
from Institutes and Special Schools
in Java, Indonesia

Farmaditya EP Mundhofir

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Genetic-Diagnostic Survey in Intellectually Disabled Individuals from Institutes and Special Schools in Java, Indonesia

Doctoral Thesis

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A Tribute to My Mother

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List of abbreviations

AAIDD	American Association on Intellectual and Development Disabilities
ADHD	Attention deficit hyperactivity disorder
ADID	Autosomal dominant ID
APA	American Psychiatric Association
APGAR	Appearance (skin color), Pulse (heart rate), Grimace (reflex irritability), Activity (muscle tone), and Respiration
ARID	Autosomal recessive ID
ASHG	American Society of Human Genetics
BAC	Bacterial artificial chromosome
bp	Base pair
BP	Breakpoint
CD	Cognitive Disorders
CDC	United States of America Centers for Disease Control and Prevention
CEBIOR	Center for Biomedical Research
CGH	Comparative genome hybridization
CMA	Chromosomal micro array
CNV	Copy number variation
DD	Developmental delay
DECIPHER	Database of chromosomal imbalance and phenotype in humans using Ensembl Resources
DGV	Database of Genomic Variants
DNA	Deoxyribonucleic acid
DSM	Diagnostic and Statistical Manual of Mental Disorders
DSM-IV-TR	Diagnostic and Statistical Manual of Mental Disorders IV Text Revision
ECARUCA	European Cytogeneticists Association Register of Unbalanced Chromosome Aberrations
EDTA	Ethylenediaminetetraacetic acid
ESHG	European Society of Human Genetics
FISH	Fluorescence <i>in situ</i> Hybridization
FoSTes	Fork stalling and template switching
FXS	Fragile-X syndrome
HC	Head circumference
ICBS	Indonesian Central Bureau of Statistics
ICD	International Classification of Diseases
ICD	Inner canthal distance
ICF	International Classification of Functioning, Disability and Health
ID	Intellectual disability
IDD	Intellectual developmental disorder
IEMs	Inborn errors of metabolisms

Abbreviations

IPD	Inter pupillary distance
IQ	Intelligence quotient
ISCA	International Standard Cytogenomic Array
ISS	Idiopathic short stature
kb	Kilobase (thousand base pairs)
LWD	Leri-Weill Dyschondrosteosis
Mb	Megabase (million base pairs)
MCA	Multiple congenital anomalies
MLPA	Multiplex Ligation-dependent Probe Amplification
MODY	Maturity-onset diabetes of the young
MPS	Massive parallel sequencing
MR	Mental retardation
MWS	Mowat–Wilson syndrome
NAHR	Non-allelic homologous recombination
NGS	Next generation sequencing
NHEJ	Non-homologous end joining
NS-XLID	Non syndromic XLID
OCD	Outer canthal distance
OFC	Occipital frontal circumference
PAR	Pseudo autosomal region
PCR	Polymerase chain reaction
PL	Palm length
PW/AS	Prader-Willi/Angelman syndrome
qPCR	qualitative Polymerase Chain Reaction
RUNMC	Radboud University Nijmegen Medical Centre
SD	Standard deviation
SNP	Single nucleotide polymorphism
SNV	Single nucleotide variant
SRQ	Self-reporting questionnaire
SSM	Slipped strand mispairing
THL	Total hand length
UPD	Uniparental disomy
UV	Unclassified variants
WES	Whole exome sequencing
WHO	World Health Organization
WHSCR	Wolf–Hirschhorn syndrome critical region
XCI	X-chromosome inactivation
XLID	X-linked intellectual disability

Chapter 1

General introduction, materials and methods, aims and outline of the thesis

1.1 General introduction

- 1.1.1 Definition, classification and epidemiology of intellectual disability (ID)
- 1.1.2 Aetiology of intellectual disability
- 1.1.3 Genetic causes of intellectual disability
- 1.1.4 Neurobiology of genetically based intellectual disability
- 1.1.5 Indonesia as a field of study

1.2 Materials and methods

- 1.2.1 Selection of patients and general procedures
- 1.2.2 Standardized clinical examination
- 1.2.3 Cytogenetic and molecular investigations
- 1.2.4 Ethical consideration

1.3 Aims and outline of the thesis

1.1.General introduction

1.1.1 Definition, classification and epidemiology of intellectual disability (ID)

Intellectual disability (ID) is a lifelong disability with major impact on individuals' lives and their families, and is a prevalent disorder worldwide. It has been estimated that the lifetime cost for medical care of a child born with an ID in the United States is roughly as high as US\$ 80.000 (Patel et al., 2010; PCPID, 2012). In Indonesia, the lifetime cost of people with ID is not known yet. However, in 2010 the amount of Rp. 3,627 billion (approximately USD 416 million) was allocated from a state budget for people with disability (Irwanto et al., 2010). Having estimated that ID is ~12% of the population of people with disability (Marjuki, 2010; Irwanto et al., 2010), the Indonesian yearly budget for taking care of people with ID is about USD 50 million.

The high cost of ID is not only a burden for the society, but for the families as well (Doran et al., 2012). The burden could even get worse if ID is compounded with other disorders, as individuals with ID are at greater risk of developing secondary health problems (Maiano, 2011). Some reports highlighted the existence of co-morbidity of ID and other conditions such as mental disorders and obesity (Einfeld et al., 2011; Maiano, 2011).

The terminology to describe ID has changed from idiocy, feeble mindedness, oligophrenia, mental deficiency, mental subnormality to mental retardation (MR). Over the last decade, an intensive discussion took place on how to properly name, define and assess ID (Salvador-Carulla et al., 2011). Nowadays, the term ID widely replaces the previous terminologies for policy, administrative and legislative purposes (Schalock et al., 2007; Salvador-Carulla et al., 2011). In 2010, the United States president Barack Obama signed into law S.2781, known as "Rosa's Law", which changes references in Federal statutes. The former term "mental retardation" is since then referred to as "intellectual disability" (van Bokhoven, 2011).

ID is a large and heterogeneous collection of syndromic and nonsyndromic disorders, highly diverse in terms of both cognitive and non-cognitive functions, multifaceted and defined in various ways, thus a comprehensive definition is difficult to give. The most widely used definition is provided by the Diagnostic and Statistical Manual of Mental Disorders IV Text Revision (DSM-IV-TR) which was formulated by American Psychiatric Association (APA) (American Psychiatric Association and Task Force on DSM-IV, 2000). Other definitions have been given by the American Association on Intellectual and Development Disabilities (AAIDD) and the World Health Organization's International Classification of Diseases (ICD-10) (Luckasson et al., 2002). In general, all definitions include a significant limitation in both intellectual

functioning (IQ<70) and concurrent limitations in conceptual, social and practical adaptive skills, and manifesting before the age of 18.

ID is subdivided into several classes on the basis of intelligence quotient (IQ) score. The DSM-IV-TR and ICD-10 subdivided it into four different degrees: mild, moderate, severe and profound. Table 1.1 presents classification of ID based on DSM-IV-TR and ICD-10. The AAIDD classification focuses on the intensity of support required by the individual highlighted into overall impact of ID in daily life. Consequently, this classification is subdivided into four groups: intermittent, limited, extensive and pervasive.

Table.1.1 Levels of intellectual disability as classified in DSM-IV-TR and ICD-10

	DSM-IV-TR *)		ICD-10	
Level of ID	Classification	IQ Level	Classification	IQ Level
Mild	317	50-55 to 70	F70	50-69
Moderate	318.0	35-40 to 50-55	F71	35-49
Severe	318.1	20-25 to 35-40	F72	20-34
Profound	318.2	<20-25	F73	<20

*) The DSM-IV-TR considers a measurement error of approximately five points, as it may occur in intelligence tests.

Adapted from American Psychiatric Association and Task Force on DSM-IV, (2000); Luckasson et al., (2002)

It has to be considered that the ID definitions given by APA, AAIDD or WHO are mainly to highlight the amount of support needed for individuals with ID in order to succeed in society, rather than to point out the degrees of impairment. Nevertheless, in terms of epidemiological studies, those definitions impose various problems. For instance, ID individuals who die in the prenatal period or during infancy are not calculated and not included in epidemiological studies. Another problem is the disagreement by what “general intellectual functioning’ exactly means and moreover there is no available validated test for it. In addition, no single measurement of adaptive behaviour that measures the ten skill domains is available (Leonard and Wen, 2002; Regan and Willatt, 2010). Therefore, a newly designed definition which is expected to overcome these problems is needed.

In an effort to overcome those problems, APA will launch the newly designed definition for ID in the Diagnostic and Statistical Manual of Mental Disorders 5 (DSM-5). This manual will be available in the middle of 2013. However, the online version can be accessed on their official website (American Psychiatric Association, 2012). The proposed definition is described below:

Intellectual Developmental Disorder (IDD) is a disorder that includes both a current intellectual deficit and a deficit in adaptive functioning with onset during the developmental period. The following 3 criteria must be met:

- A. Intellectual Developmental Disorder (IDD) is characterized by deficits in general mental abilities such as reasoning, problem-solving, planning, abstract thinking, judgment, academic learning and learning from experience.
- B. Impairment in adaptive functioning for the individual's age and sociocultural background. Adaptive functioning refers to how well a person meets the standards of personal independence and social responsibility in one or more aspects of daily life activities, such as communication, social participation, functioning at school or at work or personal independence at home or in community settings. The limitations result in the need for ongoing support at school, work, or independent life. Independent life requires self-management across life settings such as personal care, job responsibilities, financial management, recreation, managing one's behaviour, and organizing school and work tasks.
- C. All symptoms must have an onset during the developmental period.

From the definition described above, the main changes are:

1. **Name changing.** The term mental retardation was used in earlier DSM definitions including the currently used DSM-IV-TR. Nowadays, that term is no longer used and changed into Intellectual Disability (ID). In order to conform International Classification of Diseases (ICD) by World Health Organization (WHO), and as it is thought that ID is a functional disorder, the new name, IDD, is proposed (American Psychiatric Association, 2012).
2. **IQ testing**
In order to meet the criteria of the proposed ICD-11, the DSM-5 proposal does not list IQ test score requirements in the formal diagnostic criteria. This adjustment is necessary to focus on the clinical entity of IDD. Furthermore, the IQ test score has often been used improperly to identify a person's overall ability particularly in forensic cases without adequately considering adaptive functioning. Nevertheless, DSM-5 proposal continues to specify that standardized psychological testing must be included in the assessment of affected persons (American Psychiatric Association, 2012).
3. **Onset**
The onset is no longer strictly below 18 years old but symptoms should be present during the developmental period. However, there is no further explanation.

The prevalence of ID across countries and regions ranges from 2 to 85/1000 (Roeleveld et al., 1997). This range is much more obvious for mild ID (IQ 70–50) than for severe ID (IQ<50) (Ropers, 2010). In many studies, the prevalence of ID (IQ<70) has been estimated as being close to 3% worldwide (Leonard and Wen, 2002; Ropers, 2010). This figure might be particularly true for Western countries such as USA and European countries. In developing countries, the prevalence of ID is likely to be higher (Durkin, 2002). This observation has been attributed to a variety of non-genetic factors. In addition, parental consanguinity is identified as another important aetiological factor, which is particularly true in countries where consanguinity is still a common practice (Durkin, 2002). These factors make the prevalence of ID in the developing countries approximately two to threefold higher than in high income countries. It has to be considered that, in low income countries, such factors remain as a contributing causes of ID; however, these factors are largely prevented in developed countries (Ropers, 2010).

There are a number of differences in the reported prevalence of ID. This is likely occurred due to differences in the ascertainment of sources and methods (Leonard and Wen, 2002). First, the lack of consensus in conceptualization, classification and terminology are plausible factors which contribute to these differences (Fryers, 1992). Second, various methods of measuring IQ level (IQ tests) might also play a role in the variation. Third, ID is often under-detected particularly when it is associated with neuropsychiatric disorders such as epilepsy, schizophrenia and autism (Ropers, 2010). Finally, a contributing source of the variation could be because of prevalence estimation from population screening was steadily higher than those actually obtained in registers (Ropers, 2010).

1.1.2 Aetiology of intellectual disability

Establishing the aetiology of ID is of great benefit for management and genetic counselling of affected individuals and their families. A proper diagnosis may lead to appropriate management, early screening for co-morbidity and prevention of unnecessary tests; family members may take advantage of knowledge of the recurrence risk, availability of reproductive counselling and possible prenatal diagnosis. The aetiology of ID is complex and includes a wide range of factors such as biochemical/metabolic defects, chromosomal abnormalities, mutations in single genes, multifactorial disorders with a polygenic predisposition, and non-genetic causes (Chiurazzi and Oostra, 2000; van Karnebeek et al., 2005). There are various non-genetic factors associated with ID, such as the lack of maternal education, iodine deficiency in the mother or child, absence/lack of adequate prenatal and/or perinatal care, neonatal infection, lack of immunization, postnatal brain infection, postnatal

traumatic brain injury and malnutrition (Durkin, 2002). Apparently, these factors play a role as causes of ID in Indonesia. Although there is no clear data of their contribution, the prevalence of these factors in the society is still quite high compared to other countries. For instance, there are some large areas with iodine deficiency (Hussein, 1998). Equally, neonatal infection and malnutrition rates are high (Thaver and Zaidi, 2009; Pakasi et al., 2009; Oddo et al., 2012). Furthermore, pre-, peri-, and postnatal care is poor (Titaley et al., 2010) and low birth-weight commonly occurs (Amiruddin and Yusuf, 2008).

It has been estimated that at least 60% of ID cases have an underlying genetic aetiology (Battaglia and Carey, 2003; Lundvall et al., 2012). This is particularly true in cases of moderate-severe ID where a single recognizable factor is more often present (Flint and Wilkie, 1996; Lundvall et al., 2012). On the other hand, most of the cases with mild ID are generally thought to be the result of the interaction of genetic and non-genetic factors (Patel et al., 2010; van Bokhoven, 2011). That explains why nearly 60% of the individuals with ID remain without a proper diagnosis, even after intensive investigations (Moog, 2005; Patel et al., 2010; Pfundt and Veltman, 2012).

A comprehensive study on the aetiology was performed in 10,997 ID individuals by Stevenson et al. (2003). Specific causes for ID were found in 43.5% (summarized in Table 1.2.). In 2006, a study on the diagnostic yield of various genetic approaches involving 670 patients with DD/ID generated an abnormality detection rate of up to 40% (Rauch et al., 2006). The abnormalities detected in this study are summarized in Table 1.3. Since then, no similar large scale studies have been reported. Although the above studies provide a general idea on the frequencies of the main causative categories, the incidences might have changed now. Advanced technologies (high-throughput and/or high-resolution) being used to detect genetic abnormalities have facilitated the detection of many new syndromes and genetic defects. Therefore, an update on the detection rate including these novel techniques is required in the near future.

Table 1.2. Aetiology of intellectual disability adapted from Stevenson et al. (2003).

Aetiology	Frequency
Chromosomal	11.1%
Single gene	7.8%
Culturofamilial *)	5.5%
Multifactorial	1.8%
Injury	4.8%
Infection	4.7%
Prematurity	4.5%
Chemical	1.5%
Other genetic syndromes	0.8%
Syndromes presumed to be genetic	0.6%
Other environmental	0.4%
Unknown	56.5%
Total	100%

*) both culturofamilial and multifactorial were described to be the conditions caused by a combination of genetic and environmental factors (Stevenson et al., 2003). The distinguishing boundary between the two is not obviously clear. The term "culturofamilial" must be used with caution and awareness of its limitations as it does lack causality and clarity (Moog, 2005).

Table 1.3. Diagnostic yield of various methods in DD/ID population adapted from Rauch et al. (2006)

Genetic aetiology	Frequency
Numerical chromosome abnormalities	11.3%
Array technique	6.6 %
Microdeletion syndromes	5.3%
Monogenic	4.8%
Segmental chromosome aneusomy	4.7%
Mendelian	2.7%
Clinical recognizable syndromes	1.3%
Exogenic	1.3%
Submicroscopic subtelomeric	1.3%
Balanced translocations	0.6%
Mosaic trisomies	0.6%
Uniparental disomy (UPD)	0.3%
Unknown	59.2%
Total	100%

In recent years, new techniques allowed the discovery of numerous new monogenic disease genes as well as submicroscopic deletion and duplication syndromes (Mefford, 2009; Ropers, 2010; Mefford et al., 2012). By combining the targeted high-resolution approach of the fluorescence *in situ* hybridization (FISH) technology and the whole-genome approach of karyotyping, the DNA microarray technologies led to a significant change of human genome analysis (Pfundt and Veltman, 2012).

The most important platforms to detect copy number variants are comparative genome hybridization (CGH) array and single nucleotide polymorphism (SNP) arrays. The first array CGH experiments in patients with ID and dysmorphic features were based on the use of homemade DNA from bacterial artificial chromosome (BACs) spotted on glass slides (Vissers et al., 2003). Nowadays, a range of oligonucleotide-based SNP microarrays are commonly used to replace the BAC based arrays that have largely disappeared (Schaaf et al., 2011). The DNA microarray technology has proven to be successful to define new submicroscopic aberration syndromes. The use of chromosomal micro array (CMA) has a greater diagnostic yield than routine G-banded karyotyping (Sagoo et al., 2009; Miller et al., 2010). Therefore, the International Standard Cytogenomic Array (ISCA) Consortium as well as American Society of Human Genetics (ASHG) and European Society of Human Genetics (ESHG) suggested the use of CMA as the first tier cytogenetic diagnostic test for patients with developmental delay (DD)/ID, autism and multiple congenital anomalies (MCA), while the G-banded karyotyping and FISH should be kept for patients with obvious chromosomal syndromes (i.e. Down syndrome), a history of multiple miscarriages and a family history of a chromosomal rearrangement (Miller et al., 2010). Still, these techniques are solely meant for the detection of chromosomal imbalances and are not suitable for the detection of most monogenic causes of ID.

Lately, a new technique named massive parallel sequencing (MPS) or next generation sequencing (NGS) enables sequencing of millions of DNA fragments in a parallel fashion, allowing the possibility to detect all kinds of genomic variation in a single experiment (Gilissen et al., 2012). One of the most promising new methods for disease gene identification utilizing the NGS technology is exome sequencing. Exome sequencing allows the study of the protein-coding part of the genome from an individual in a single experiment (Vissers, 2010a; Gilissen, 2012; Majewski, 2012).

1.1.3. Genetic causes of intellectual disability

Chromosomal abnormalities

Pathogenic chromosomal abnormalities are considered to be the most common genetic cause of ID (Stevenson et al., 2003; Rauch et al., 2006; Lundvall et al., 2012). After the fundamental discovery that normal human cells contained 46 chromosomes in 1956 (Tjio and Levan, 1956), it was later followed by identification of numerical chromosome aberrations in individuals with ID/MCA such as Down syndrome (trisomy 21) (LeJeune et al., 1959), Turner syndrome (45,X) (Ford et al., 1959), Klinefelter syndrome (47,XXY) (Jacobs and Strong, 1959), Patau syndrome (trisomy 13) (Patau et al., 1960) and Edward syndrome (trisomy 18) (Edwards et al., 1960). In addition, several chromosomal abnormalities like unbalanced translocations, supernumerary marker chromosomes and large (>5 Mb) deletions and duplications were later identified and recognized as causes of ID (Smeets, 2004). The microscopically visible numerical and structural abnormality rates vary considerably among different studies. A rate between 7–56% was reported, reflecting differences between techniques and patient selection (van Karnebeek et al., 2002; Dayakar et al., 2010). In a large meta-analysis, a median rate of 9.5% was found (van Karnebeek et al., 2005).

Down syndrome (trisomy 21) is the most common classical chromosomal aneuploidy causing ID. It is the most frequent genetic form of ID with a relatively constant frequency of 9-12% (Leonard and Wen, 2002; Stevenson et al., 2003). The prevalence of other microscopically visible aberrations is less common. If patients with Down syndrome were excluded, the cumulative frequency of such aberration is fewer than 5% (Rauch et al., 2006; Miller et al., 2010; Lundvall et al., 2012).

During the last decade, molecular techniques became available to identify submicroscopic cytogenetic abnormalities (Trask, 2002; Smeets, 2004). FISH, targeted qualitative Polymerase Chain Reaction (qPCR) and Multiplex Ligation-dependent Probe Amplification (MLPA) allowed identification of submicroscopic rearrangements (Flint et al., 1995; Knight et al., 1999; Schouten et al., 2002; Hoebeeck et al., 2005). Some common cryptic rearrangements lead to recurrent submicroscopic abnormalities such as Prader-Willi and Angelman syndromes (15q11.2-q13), Williams-Beuren syndrome (7q11.23), Smith-Magenis syndrome (17p11.12) and DiGeorge syndrome (22q11.2) (Shaffer et al., 2007a; Gropman and Batshaw, 2010). The prevalence of such abnormalities was estimated to be about 4% (Rauch et al., 2006).

Apart from those common cryptic rearrangements, segmental aneusomies are a common cause of ID and about half of these include a telomere (Biessacker, 2002). After Flint et al. (1995) found subtelomeric microdeletions using FISH in unrelated

patients with ID and helped by the upcoming availability of molecular probes specific for the region, many studies attempted to determine the prevalence of such conditions in the ID population (Knight et al., 1999; Baker et al., 2002). Biesecker reviewed 14 previously reported studies with different methods and ascertainment involving 1718 subjects that performed submicroscopic subtelomeric analysis from 1995-2002. That review generated a mean abnormality rate of 6% with a range of 2–29% in individual studies (Biesecker, 2002). Several studies later identified an abnormality rate of subtelomeric submicroscopic rearrangements in the range of 5-9% (Christofolini et al., 2010; Wu et al., 2010; Jehee et al., 2011).

Various assays such as FISH, MLPA and qPCR to identify smaller cytogenetic abnormalities, however, are limited to specific regions, e.g. subtelomeric region, and cannot identify submicroscopic imbalances elsewhere. DNA microarray technology combines the targeted high resolution approach of FISH/MLPA/qPCR technology and the whole genome approach of conventional karyotyping technology (Vissers et al., 2010b; Pfundt and Veltman, 2012). Therefore, it allows the detection of submicroscopic genomic variation at a resolution far below 5 Mb as well as enables wide screening for submicroscopic rearrangements (Menten et al., 2006; Bejjani and Shaffer, 2006). This new technology has led to an aetiological diagnosis in another ~14% of ID cases (Miller et al., 2010; Cooper et al., 2011). Nowadays, the DNA microarray technology supersedes the use of conventional karyotyping and other previous assays such as FISH, MLPA and qPCR in diagnostic laboratories in western countries. This technology, however, is hardly available in developing countries such as Indonesia.

Submicroscopic interstitial chromosomal imbalances are responsible for a considerably number of ID cases. Such rearrangements involve chromosomal deletions/duplications that span several genes but are too small to be detected using conventional cytogenetic analysis (Shaffer et al., 2007a). These few common rearrangements in this size-range are also referred to chromosome microdeletion/microduplication genomic disorders (Lupski, 2009; Vissers and Stankiewicz, 2012). Apart from deletions and duplications, such rearrangements may include insertions, inversions and balanced translocations. These rearrangements are caused by regional genomic architecture which may lead to the gain or loss of a dosage sensitive gene (or genes) or disruption of a gene (or genes) (Vissers et al., 2009). There are three major underlying mechanisms that may lead to copy number changes namely non-allelic homologous recombination (NAHR), non-homologous end joining (NHEJ) and fork stalling and template switching (FoSTes) (Hastings et al., 2009; Lupski, 2009; Zhang et al., 2009). Overall, such rearrangements fall into two categories, namely recurrent and non-recurrent aberrations. While recurrent rearrangements are characterized by a common size and clustered breakpoints, non-

recurrent ones are usually unique and spread over a certain genomic region (Vissers et al., 2009).

Formerly, recognition of submicroscopic aberration syndromes relied on identification of chromosome abnormalities in a collection of patients with a common phenotype and delineation of a critical genomic region due to an initial observation of a balanced or unbalanced translocation (Shaffer et al., 2007a). For such rearrangements, identification of the cytogenetic origin was first established by detection of a common aberration in a cohort of individuals with similar phenotypic features, e.g. the identification of 15q12 deletion in Prader–Willi syndrome (Ledbetter et al., 1981). The other famous examples include Williams-Beuren and Miller-Dieker syndrome (Dobyns et al., 1983; Ewart et al., 1993; Lowery et al., 1995; Brewer et al., 1996). Nowadays, the opportunity to perform genome wide copy number variation (CNV) analysis in individuals with ID has significantly increased the possibility to find new submicroscopic aberration syndromes (Shaffer et al., 2007a; Shaffer et al., 2007b). This led to a shift from a “phenotype first” to a “genotype first” or “reverse phenotypic” approach in which patients are identified by a similar genomic aberration before a common clinical presentation is defined (Shaffer et al., 2007b). Several submicroscopic aberration syndromes associated with ID and/or related disorders are summarized in Table 1.4.

Table 1.4. Several submicroscopic aberration syndromes associated with ID and/or related disorders (adapted from Ropers, 2010; Vissers et al., 2010b; Vissers and Stankiewicz, 2012)

Genomic region/name	MIM	Region of overlap (Mb)	Clinical features	Selected references
1q21 microdeletion	612474	1.2	Mild to moderate intellectual disability, schizophrenia, autism, microcephaly, cataracts, cardiac abnormalities	(Mefford et al., 2008; Stefansson et al., 2008)
1q21 microduplication	612475	1.2	Risk factor for intellectual disability and autism spectrum disorder, macrocephaly, congenital abnormalities	(Szatmari et al., 2007; Brunetti-Pierri et al., 2008)
2p15p16.1	612513	4.0	Moderate to severe intellectual	(Rajcan-Separovic et

Genomic region/name	MIM	Region of overlap (Mb)	Clinical features	Selected references
microdeletion			disability, microcephaly, optic nerve hypoplasia, hydronephrosis, facial abnormalities, feeding problems, camptodactyly	al., 2007; de Leeuw et al., 2008)
3q29 microdeletion	609425	1.8	Mild to moderate intellectual disability, autism, microcephaly, dysmorphic facial features, ataxia, chest wall deformity, long tapering fingers	(Willatt et al., 2005; Ballif et al., 2008)
3q29 microduplication	611936	1.8	Mild to moderate intellectual disability, microcephaly, ophthalmological problems	(Ballif et al., 2008; Goobie et al., 2008; Lisi et al., 2008)
5q35 microdeletion (Sotos syndrome)	117550	2.3	Intellectual disability, severe learning disabilities, overgrowth, macrocephaly, dysmorphic face	(Kurotaki et al., 2005; (Tatton-Brown et al., 2005)
7q11.23 microdeletion (Williams–Beuren syndrome)	194050	1.5	Intellectual disability, short attention span, aortic stenosis, small teeth, facial abnormalities, hernias	(Grimm and Wesselhoeft, 1980; Tassabehji, 2003)
7q11.23 microduplication /triplication	609757	1.5	Mild intellectual disability, autism, speech delay, seizures type II trigonocephaly, cortical dysplasia, growth retardation	(Somerville et al., 2005; Depienne et al., 2007; Torniero et al., 2008)
8p23.1 microdeletion syndrome		3.6	Intellectual disability, hyperactivity, congenital hernia of diaphragm, heart defects, cryptorchid testes	(Faivre et al., 1998)
9q22.3 microdeletion		6.5	Intellectual disability, hyperactivity, psychomotor	(Redon et al., 2006)

Genomic region/name	MIM	Region of overlap (Mb)	Clinical features	Selected references
			delay, macrocephaly, trigonocephaly, facial abnormalities, overgrowth	
15q11.2 microdeletion		0.3	Risk factor for epilepsy	(de Kovel et al., 2010)
15q13.1 microduplication			Intellectual disability, schizophrenia, autism	(van Bon et al., 2009; de Kovel et al., 2010)
15q13.3 microdeletion	612001	1.5	Risk factor for intellectual disability, schizophrenia, autism, and epilepsy, facial abnormalities, clinodactyly	(Sharp et al., 2008; Dibbens et al., 2009; Helbig et al., 2009)
15q24 microdeletion	613406	1.5	Mild intellectual disability, growth retardation, microcephaly, digital abnormalities, hypospadias, loose connective tissue, facial abnormalities	(Sharp et al., 2007; Klopocki et al., 2008)
15q24 microduplication	613406	1.5	Developmental delay, dysmorphic facial features, digital and genital abnormalities	(Kiholm Lund et al., 2008)
16p11.2 microdeletion	611913	0.7	Intellectual disability, autism, developmental disorder, motor delay, seizures, behavioral problems, speech delay, macrocephaly, congenital abnormalities	(Weiss et al., 2008; Shinawi et al., 2010)
16p11.2 microduplication	611913	0.7	Schizophrenia, bipolar disorder, autism, motor delay, speech delay, ADHD, microcephaly, congenital abnormalities	(Weiss et al., 2008; McCarthy et al., 2009; Shinawi et al., 2010)

Genomic region/name	MIM	Region of overlap (Mb)	Clinical features	Selected references
16p11.2p12 microdeletion		7.1	Intellectual disability, recurrent ear infection, minor facial abnormalities, feeding difficulties	(Ballif et al., 2007; Ghebranious et al., 2007; Hempel et al., 2009)
16p13.1 microdeletion		1.5	Intellectual disability, risk factor for epilepsy and schizophrenia, dysmorphic features	(Ullmann et al., 2007; Hannes et al., 2009; de Kovel et al., 2010)
16p13.1 microduplication		1.5	Risk factor for intellectual disability, autism, and schizophrenia	(Ikeda et al., 2010; Ingason et al., 2011)
17p13.3 microdeletion (<i>LIS1</i> gene)			Intellectual disability, epilepsy, lissencephaly	(Mei et al., 2008)
17p11.2 microdeletion (Smith–Magenis syndrome)	182290	3.5	Intellectual disability, hyperactivity, self-mutilation, sleep disorder, hoarse voice, brachydactyly, hypotonia	(Chen et al., 1997)
17p11.2 microduplication (Potocki–Lupski syndrome)	610883	3.8	Mild intellectual disability, ADHD, autism, infantile hypotonia, failure to thrive, sleep apnea, cardiovascular abnormalities	(Potocki et al., 2000; Potocki et al., 2007)
17q11.2 microdeletion (<i>NF1</i> gene)	613675	1.4	Intellectual disability, patchy skin pigmentation, hypertelorism, coarse facial features	(Dorschner et al., 2000; Kluwe et al., 2004)
17q12 microdeletion	614527	1.4	Intellectual disability, renal phenotype, elevated liver enzymes, maturity-onset diabetes of the young (MODY)	(Muller et al., 2006; Mefford et al., 2007)
17q12 microduplication	614526	1.4	Risk factors for mild to moderate intellectual disability and epilepsy	(Mefford et al., 2007)

Genomic region/name	MIM	Region of overlap (Mb)	Clinical features	Selected references
17q21.31 microdeletion	610443	0.6	Intellectual disability, learning disabilities, neonatal hypotonia, heart defects, kidney defects, coarsening of facial features with age, oromotor dyspraxia	(Sharp et al., 2006; Koolen et al., 2006; Shaw-Smith et al., 2006)
17q21.31 microduplication	613533	0.6	Intellectual disability, severe psychomotor developmental delay, poor social interaction, microcephaly, abnormal digits, hirsutism, facial dysmorphism	(Kirchhoff et al., 2007; Grisart et al., 2009)
22q11 microdeletion	611867	0.6	Mild learning disabilities, speech delay, schizophrenia, heart defects, renal abnormalities, neonatal hypocalcemia, velopharyngeal insufficiency, facial abnormalities	(Ryan et al., 1997)
22q11 microduplication /triplication	608363	3.0	Intellectual disability, learning disabilities, growth retardation, muscular hypotonia, delayed psychomotor development	(Ensenauer et al., 2003; Yobb et al., 2005; Wentzel et al., 2008)
22q11.21-q11.23 microdeletion	611867		Intellectual disability, facial abnormalities, growth delay, cardiovascular malformation, mild skeletal abnormalities	(Ben-Shachar et al., 2008)
22q11.21-q11.23 microduplication	608363		Intellectual disability, no speech or speech delay, no walking, apraxia and dysarthria, facial abnormalities	(Mefford et al., 2009; Coppinger et al., 2009)
22q13 microdeletion	606232	0.1	Global developmental delay, absent to severely delayed speech, autistic-like behavior,	(Phelan, 2008; Mefford et al., 2009)

Genomic region/name	MIM	Region of overlap (Mb)	Clinical features	Selected references
			minor dysmorphic features	
Xp22.31 microdeletion	308100		Intellectual disability, X-linked ichthyosis	(van Esch et al., 2005b)
Xp22.31 microduplication			Intellectual disability, speech delay, microcephaly	(Wagenstaller et al., 2007)
Xq28 microduplication		0.4-0.8	Severe intellectual disability, severe hypotonia, speech delay, seizures, recurrent infections	(van Esch et al., 2005a)

X-Linked intellectual disability

The excessive number of males over females among all severities of ID has been noticed since a long time ago. The difference is ranging from 30% to 50% and is consistent among various populations. This suggests that X-linked genes play a role in the aetiology of ID (Hussein, 1998; Ropers and Hamel, 2005; Lundvall et al., 2012). In the early 1930s, the investigation referred as Colchester Survey revealed that males outnumbered females among a wide range of ID population. This study included 1280 institutionalized individuals with all severities of ID (Penrose, 1938; Morton et al., 1977). In the 1940s, Martin and Bell described a condition with “sex-linked” ID which is now known to be FXS. Such investigation illustrated that “sex-linked” inheritance was the explanation for the presence of ID in a two generations family with 11 affected males (Martin and Bell, 1943). In the early of 1970s, Lehrke was the first to argue for the existence of major genes related to intellectual function situated on the X chromosome. In addition, Lehrke put forward the four hypotheses: i) there are major genetic loci associated with human intellectual functioning which are located in the X chromosome; ii) mutation on these genes can lead to subnormal intellectual function; iii) one or more of these genes is particularly associated to verbal functioning; and iv) the deficit links primarily to the central nervous system (Lehrke, 1972; Lehrke, 1974).

Still in the 1970s, the publication of the first family with the marker X by Lubs et al. (1969) gave an important momentum to the field by offering a laboratory tool which clearly identified the most prevalent XLID syndrome (Lubs, 1969). Several years later, linkage studies revealed that the location of the marker chromosome (fragile site)

overlapped with the map position of underlying genetic defect (Verkerk et al., 1991). These findings paved the way for molecular elucidation of the FXS and since then FXS remained the centre of research into ID which triggered the findings of other XLID genes (Ropers, 2010; Lubs et al., 2012).

In the past decade, X chromosome sequencing, DNA microarrays technology and exploration of molecular pathways have enhanced the elucidation of even more XLID genes (Gecz et al., 2009; Lubs et al., 2012). The application of powerful DNA microarray technology in combination with various molecular technologies has progressively been used to identify duplications and deletions of genes associated with XLID (Froyen et al., 2007; Tarpey et al., 2009; Whibley et al., 2010; Gijbbers et al., 2011). Up to now, more than 150 syndromes associated with XLID have been described, with FXS as the most common one (Lubs et al., 2012; Stevenson et al., 2012). In addition, a total of 95 families with NS-XLID has been regionally mapped. Furthermore, mutations in 102 X-linked genes have been associated with 81 out of 160 XLID syndromes as well as 50 families with NS-XLID. Moreover, an additional 30 XLID syndromes and 48 families with nonsyndromal XLID have been regionally mapped in which the genes have not yet been identified (Lubs et al., 2012).

Autosomal forms of intellectual disability

Since X-linked forms account for only 10-15% of ID cases, the vast majority of the underlying genetic defects are believed to be autosomal either dominant (autosomal dominant ID; ADID) or recessive (autosomal recessive ID; ARID) (Ropers, 2010; van Bokhoven, 2011). It is estimated that more than 800 genes associated with ID are located in the autosomes (Ropers, 2010). For several reasons, the elucidation of these autosomal genes has been lagging far behind the XLID. The major reason is the lack of suitable families for accurate mapping of genetic defects. Lately, significant progress in elucidating autosomal ID genes has been made. However, still little is known about the gene defects underlying these autosomal forms (Ropers, 2010; van Bokhoven, 2011). Families with autosomal dominant inheritance are rare since affected individuals in general will not reproduce. Strategies to identify autosomal dominant genes, therefore, direct to the identification of *de novo* mutations (Ropers, 2007).

Until 2007, no more than twelve loci had been mapped for nonsyndromic ARID (Basel-Vanagaite, 2007). In addition, only three genes had been identified, namely *PRSS12* (Molinari, 2002), *CRBN* (Higgins et al., 2004), and *CC2D1A* (Basel-Vanagaite et al., 2006), all in large consanguineous families. Nowadays, several strategies to elucidate the molecular defects underlying ARID have been utilized. For instance, homozygosity mapping in large consanguineous families followed by mutation screening of candidate genes has been employed to identify several gene defects of

ARID (Ropers, 2007). In general, this strategy will identify one or a few regions of shared homozygous haplotypes, ranging from a few to several Mb per family (van Bokhoven, 2011). Another strategy is using a massive parallel sequencing approach. This recent innovation has expedited the successful identification of causative mutations in autosomal ID (van Bokhoven, 2011). Recently, by combining homozygosity mapping, exon enrichment and NGS in 136 consanguineous families with ARID, 50 novel candidate genes for ARID have been revealed (Najmabadi et al., 2011). This study is the largest published so far in the field of autosomal form of ID.

De novo mutations refer to mutations which occur in the gametes of the parents during meiosis. It is estimated that humans have an exceptionally high per-generation mutation rate of between 7.6×10^{-9} and 2.2×10^{-8} . Therefore, it is calculated that an average newborn would acquire 50–100 new mutations in his or her genome (Roach et al., 2010; Lynch, 2010). The study of *de novo* point mutations remained stagnant until the advent of NGS. Vissers et al. (2010a) first demonstrated, by using a family based whole-exome sequencing (WES - sequencing of all the exons in the genome) approach, that *de novo* mutations are a common cause of ID. Ten case-parent trios were sequenced in order to investigate potentially pathogenic *de novo* changes in the exome sequences of the affected offspring with otherwise normal karyotype and array-based genome profiling. In addition to ID, *de novo* mutations have also been found to have an important role in other neurodevelopmental disorders such as autism (O'Roak et al., 2011) and schizophrenia (Xu et al., 2011). Subsequently, a number of *de novo* mutations underlying mendelian rare disorders associated with neurodevelopmental defects were identified using WES technology (summarized in Table 1.5).

Table 1.5 *De novo* mutations underlying rare syndromic ID identified by whole-exome sequencing studies (adapted from Ku et al., 2012)

Disorder	MIM	Associated clinical features	Gene	References
Kabuki syndrome	147920	Mild-to-moderate intellectual disability, distinctive facial appearance, cardiac anomalies, skeletal abnormalities and immunological defects.	<i>MLL2</i>	(Ng et al., 2010)

Disorder	MIM	Associated clinical features	Gene	References
Schinzel–Giedion syndrome	269150	Severe intellectual disability, distinctive facial features and multiple congenital malformations (including skeletal abnormalities, genitourinary and renal malformations and cardiac defects).	<i>SETBP1</i>	(Hoischen et al., 2010)
Bohring–Opitz syndrome	605039	Severe intellectual disability, prominent metopic suture, exophthalmus, forehead nevus flammeus, upslanting palpebral fissures, ulnar deviation and flexion of the wrists and metacarpophalangeal joints, and severe feeding problems.	<i>ASXL1</i>	(Hoischen et al., 2011)
Say-Barber-Biesecker-Young-Simpson syndrome / Ohdo syndrome	603736	Severe intellectual disability, blepharophimosis, a mask-like facial appearance, thyroid abnormalities and cleft palate.	<i>KAT6B</i>	(Clayton-Smith et al., 2011)
Hypothyroidism	614450	Hypothyroidism (growth retardation, develop intellectual disability, skeletal dysplasia and severe constipation) but only borderline-abnormal thyroid hormone levels.	<i>THRA</i>	(Bochukova et al., 2012)
Genitopatellar syndrome	606170	Severe intellectual disability, patellar aplasia or	<i>KAT6B</i>	(Campeau et al., 2012;

Disorder	MIM	Associated clinical features	Gene	References
		hypoplasia and external genital anomalies.		Simpson et al., 2012)
KBG syndrome	148050	Intellectual disability, macrodontia of the upper central incisors, distinct craniofacial findings, short stature and skeletal anomalies.	<i>ANKRD11</i>	(Sirmaci et al., 2011)
Weaver syndrome	614421	Generalized overgrowth, advanced bone age, marked macrocephaly, hypertelorism and characteristic facial features. Intellectual disability is common.	<i>EZH2</i>	(Gibson et al., 2012)
Floating-Harbor syndrome	136140	Short stature, delayed osseous maturation, expressive-language deficits and a distinctive facial appearance.	<i>SRCAP</i>	(Hood et al., 2012)
Hereditary diffuse leukoencephalopathy with spheroids	221820	White-matter abnormality and variable clinical presentations (personality and behavioural changes, dementia, depression, parkinsonism, seizures and other phenotypes).	<i>CSF1R</i>	(Rademakers et al., 2012)
Coffin–Siris syndrome	135900	Developmental delay, severe speech impairment, coarse facial features, hypertrichosis, hypoplastic or absent fifth fingernails or toenails and agenesis of the corpus callosum.	<i>ARID1B</i> <i>SMARCB1</i>	(Tsurusaki et al., 2012; Santen et al., 2012)

Disorder	MIM	Associated clinical features	Gene	References
Olmsted syndrome	607066	Bilateral mutilating palmoplantar keratoderma and periorificial keratotic plaques with severe itching at all lesions.	<i>TRPV3</i>	(Lin et al., 2012)
Baraitser–Winter syndrome	614583 243310	Intellectual disability, seizures, hearing loss, congenital ptosis, high-arched eyebrows, hypertelorism, ocular colobomata and a brain malformation consisting of anterior predominant lissencephal, postnatal short stature and microcephaly.	<i>ACTB</i> <i>ACTG1</i>	(Riviere et al., 2012)
Autosomal-dominant cerebellar ataxia, deafness and narcolepsy	604121	Late onset (age 30–40 years) narcolepsy–cataplexy, sensory neuronal deafness, cerebellar ataxia, dementia, psychosis, optic atrophy and other symptoms.	<i>DNMT1</i>	(Winkelmann et al., 2012)
Mandibulofacial dysostosis with microcephaly	610536	Craniofacial malformations, microcephaly, developmental delay, recognizable dysmorphic appearance, choanal atresia, sensorineural hearing loss and cleft palate.	<i>EFTUD2</i>	(Lines et al., 2012)
Myhre syndrome	139210	Reduced growth, generalized muscular hypertrophy, facial	<i>SMAD4</i>	(Caputo et al., 2012)

Disorder	MIM	Associated clinical features	Gene	References
		dysmorphism, deafness, cognitive deficits, joint stiffness and skeletal anomalies.		

Metabolic disturbances

Inborn errors of metabolisms (IEMs) are possible treatable causes of ID (van Karnebeek and Stockler, 2012). Using routine metabolic investigations, such causes can be identified in 0.2 to 8.4% of ID cases (van Karnebeek et al., 2005; Sempere et al., 2010). Moreover, detailed and tailored metabolic assessment yielded causative IEMs up to 20% cases in a population with unexplained neurodevelopmental disorders that were referred to a tertiary diagnostic centre (Engbers et al., 2008). To date, there are 81 IEMS with ID as a major clinical feature (van Karnebeek and Stockler, 2012). Although the prevalence of each of the 81 conditions is considered low, varying from 1:10,000 – 1:200,000, detection is important because of its possible treatability (van Karnebeek and Stockler, 2012). Albeit that evidence for the majority of the therapies is limited, it is reported that in clinical practice more than 60% of treatments have 4–5 evidence level, an internationally accepted level as ‘standard of care’ (van Karnebeek and Stockler, 2012). A list of 81 IEMs and its availability of treatment is available in www.treatable-id.org.

1.1.4. Neurobiology of genetically based intellectual disability

There are several general molecular and cellular mechanisms underlying the pathophysiology of genetically based ID. In general, they can be subdivided into two groups, those with detectable cortical brain developmental abnormalities and those with an apparent normal brain organization (Chelly et al., 2006; Vaillend et al., 2008). In many circumstances where detectable developmental brain abnormalities are present, ID is a component of a complex syndrome where neurodevelopmental programmes and normal organizational patterns of cerebral cortex are disrupted and thus lead to developmental brain abnormalities (Chelly et al., 2006; Francis et al., 2006). In these conditions, ID is presumably a secondary symptom (Chelly et al., 2006). On the contrary, for those with an apparent normal brain organization and architecture, ID is most likely due to abnormal interaction of neuronal and/or glial cells (Chelly et al., 2006;

Vaillend et al., 2008). Mutations in some genes are associated with a variety of different Cognitive Disorders (CDs). This may be an explanation for the high comorbidity that is commonly observed between ID and other cognitive impairments, such as autism, attention deficit hyperactivity disorder (ADHD) and schizophrenia (van Bokhoven, 2011).

Functional studies have shown that these genes are involved in neurodevelopmental processes and share common signaling pathways, highlighting their significance for normal cognitive development. In addition, it is evident that, for example, even mild alterations in synapse morphology and function can cause ID (Vaillend et al., 2008; van Bokhoven, 2011; Verpelli and Sala, 2011). Interestingly, it seems that some pharmacological agents are able to work against these morphological and functional anomalies. This may lead to the feasibility of future targeted treatment (Vaillend et al., 2008; van Bokhoven, 2011; Verpelli and Sala, 2011).

1.1.5. Indonesia as a field of study

The population of Indonesia in 2010 reached 237,641,326 people. Among countries with the largest population in the world, Indonesia ranks the fourth after China, India, and the United States (Indonesian Central Bureau of Statistics, 2011a). To date, there are little data on prevalence and aetiology of ID in Indonesia. There are four data sources for the ID individual: Indonesian Central Bureau of Statistics (ICBS), the Indonesian Ministry of National Education, the Indonesian Ministry of Social Affairs and the Indonesian Ministry of Health. Based on house-to-house direct population census, ICBS stated that a total of 4,785,876 (~2% of the population) individuals were reported to have difficulties in physical and psychological functioning areas including remembering, concentrating, communicating, and taking care of themselves (Indonesian Central Bureau of Statistics, 2011b). Among them, 1,149,078 were reported to be in severe conditions, requiring full support in all aspects of daily living (Indonesian Central Bureau of Statistics, 2011c). The Indonesian Ministry of National Education stated that only approximately 66,500 individuals were registered in special schools for intellectually disabled (Kemendiknas, 2011). However, this data cannot be used to figure out the real prevalence of ID in Indonesia as it does not include the number of those staying in formal institutions as well as those not attending school. In 2010, the Indonesian Ministry of Social Affairs conducted a survey on the basis of ICF classification by WHO and found 170,398 ID individuals out of 1,167,111 individuals with various disabilities. This figure was determined from only 14 out of 33 provinces targeted in the survey (Marjuki, 2010). The data of the Indonesian Ministry of Health in the *Riset Kesehatan Dasar* (Basic Health Research) project in 2007 estimated that the national prevalence of mental and emotional impairment is 11.6%, while the prevalence

of severe mental disability is 0.46% (Badan Litbangkes Depkes, 2007). This estimation, however, was calculated among people aged 15 and over and based on a self-reporting questionnaire (SRQ). Therefore, it is likely that this figure is inaccurate because it does not include those below 15 years old and very likely those with severe mental impairment could not participate in the SRQ based survey (Badan Litbangkes Depkes, 2007; Badan Litbangkes Kemenkes, 2010). It should be taken into account that numerous cases of ID had been overlooked as they were hardly found in the registers. For instance, cases of confinement (*pemasungan*—being captivated in a wooden block) of individuals who are intellectually and mentally disturbed are still put into practice in some communities (Minas and Diatri, 2008; Tyas, 2008). Furthermore, ID individuals are often being vagrant (*gelandangan*) and live on the streets.

It is regrettable that in some areas of Indonesia, individuals with ID and their families are still being stigmatized. In addition, they frequently receive inadequate services or funding (Komardjaja, 2005; Irwanto et al., 2010). Actually, legislation (Law No.4/1997) forces private companies and governmental departments to employ disabled people. However, this legislation is overlooked because the application, enforcement and sanctions of laws and regulations, in general, are weak and easily infringed (Republic Indonesia Laws No.4/1997; Komardjaja, 2005; Irwanto et al., 2010).

ID individuals can be observed in special schools, institutions and special dormitories (Komardjaja, 2005). The two latter places are important places for ID people to live in and even some parents consider these places as the 'perfect' place to hide their ID children. Families are often ashamed of having kin with ID (Komardjaja, 2005). Therefore, hiding their kin with ID in those places is common practice. Some individuals with ID ended up their whole lives in those places without being cared for and visited by their families (Komardjaja, 2005). In several cases, regrettably, some families are no longer in contact with caregivers in those places on purpose and feel no longer responsible for their kin anymore. That is why, in particular, the largest budget allocation for ID individuals is for social security and food allowance (Irwanto et al., 2010). Consequently, the relatively small remaining funding is to build infrastructure, develop human resources and develop empowerment programmes. Due to the low budget, the quality of services turns out to be problematic. One of the apparent problems is the unavailability of funding to hire professional care givers (Irwanto et al., 2010).

Medical genetics is considered to be a new area in Indonesia. In fact, this field grows fast in neighbouring countries of Indonesia such as Singapore and Australia. Nowadays, only a few medical geneticists and no clinical geneticist are available in Indonesia. Likewise, diagnostic services for genetic abnormalities are rarely found. In some big cities on Java Island such as Jakarta, Bandung, Semarang, Yogyakarta and

Surabaya, some hospitals / universities offer low resolution karyotyping, mostly limited to the diagnosis of numerical chromosomal abnormalities such as Down syndrome and sex assignment. The awareness of medical practitioners and specialists related to genetics such as paediatricians, neurologists, and obstetricians is still very low. The genetic diagnostic services built by government and/or private companies are still lagging behind the development of diagnostic facilities for infectious and tropical diseases. Concerning individuals with ID, there are only two centres which perform comprehensive evaluations: Eijkman Institute in Jakarta and the Center for Biomedical Research (CEBIOR), Faculty of Medicine, Diponegoro University in Semarang. It is obvious that the number of clinics, diagnostic facilities and human resources is far too small in comparison to the population of Indonesia.

1.2. Materials and methods

1.2.1. Patient selection, setting and characteristics

Prior to this study, our laboratory (Center for Biomedical Research, Faculty of Medicine Diponegoro University, Semarang, Indonesia) had been developing some collaborations with several schools and institutions within and outside the city of Semarang such as Temanggung, Yogyakarta and Bandung. The majority of our subjects were from schools and institutions in Semarang due to the proximity to our laboratory. As representatives from some other areas were needed as well, subjects from Temanggung, Yogyakarta and Bandung were also included. At the time of the study, 594 individuals (361 males and 233 females) were registered in those institutions (193 individuals) and schools (401 individuals). From this cohort, 527 individuals (329 males and 198 females) were investigated, leaving 23 individuals who were temporary absent and 44 individuals for whom no permission was obtained. A consecutive sampling method was used. A standardized and dysmorphological physical examination comprising physical measurements and dysmorphological assessment has been carried out in all subjects. They were residents of four different places in Java Island, Indonesia (Semarang, Temanggung, Gunung Kidul, and Bandung). Out of the 527 patients, 156 were institutionalized, while 371 came from special schools. Based on the IQ data from those schools/institutions, the majority of the individuals (345) appeared to be mildly intellectually disabled, while 161 were moderate, and the other 21 patients were severely intellectually disabled. The information about characteristics of the study population is summarized in Table 1.6

Table 1.6. Characteristics of the studied population ($n = 527$)

Demographic data		<i>n</i>
Gender		
Male		329
Female		198
Area / City		
Semarang		327
Temanggung		134
Yogyakarta		11
Bandung		55
School type		
Institution		156
Special school		371
ID severity		
Mild ID		345
Moderate ID		161
Severe ID		21
Family history		
Familial		
Sibling		10
Second cousin		4
Sporadic		513

1.2.2. Standardized clinical examination

A standardized clinical examination protocol adapted from Radboud University Nijmegen Medical Centre (RUNMC), Nijmegen was performed by the same clinician (Farmaditya EP Mundhofir) and supervised by experienced clinical geneticists from RUNMC, Nijmegen. This examination is divided into three parts comprising history taking, clinical measurements, and clinical examination including dysmorphological assessment. If available, medical history including mental functioning status was recorded. History taking comprising pre-, peri-, and postnatal history, pedigree construction and developmental history was performed when subjects' parents/caregivers were available. IQ test, either Weschler's or Binet's, is compulsory for individuals with ID prior to the admission to the special school or institution. For

several cases where exact IQ could not be determined, a range of IQ level was assigned.

Physical measurements like weight, height, head circumference (OFC), outer canthal distance (OCD), inner canthal distance (ICD), inter pupillary distance (IPD), ear length, total hand length (THL), palm length (PL), and - in males - testicular volume were documented in all individuals. Some others such as arm span and sitting height were performed if there is a suspicion of a specific condition. Clinical examination comprised the general habitus and dysmorphological assessment of craniofacial, thorax, abdomen, back, genitalia, upper and lower extremities was performed. Behaviour including general impression of the personality and some peculiar manners such as posture, movement, walking, mimic and stereotypes were observed and recorded. Finally, standard clinical pictures were taken from each subject. Due to technical and financial constraints, however, some investigations such as metabolic investigation and brain imaging were not performed.

1.2.3. Cytogenetic and molecular investigations

Peripheral duplicate blood samples in EDTA and Heparin were collected from December 2006 to November 2008. Genetic investigations comprising cytogenetic analysis, molecular analyses to identify FXS and subtelomeric rearrangements using MLPA, and specific tests to identify the presence of specific syndromes (i.e. Prader-Willi/Angelman syndrome, ID with epilepsy and ID with macro/microcephaly) were performed.

Chromosome cultures and preparations were carried out as described elsewhere (Hussein, 1998). One hundred metaphases were screened for fragile sites on each sample. Subsequently, chromosome analysis was performed by using G-banding technique at the level of 400–600 bands. At least 20 metaphases were scored for each patient and karyotyped. If a mosaicism was suspected, 50–100 cells were counted. MLPA, FISH and SNP array were carried out to confirm the presence of cytogenetic abnormalities.

Genomic DNA of each patient was isolated by using the salting-out method as described elsewhere. The CGG repeat in the *FMR1* promoter was amplified as described by Fu et al. (1991). Fragment length analysis was carried out on an ABI Prism 3730 DNA Analyzer (Life Technologies, Foster City, USA), and the Genemapper software (version 4.0, Apache) was used to determine the exact length of the CGG repeat. Southern blot analysis of the *FMR1* CGG(n) repeat was performed as previously described (Spath et al., 2010).

MLPA analysis was performed as described before (Schouten et al., 2002). Two probe-kits for subtelomeric chromosomal imbalances were SALSA P070 and

SALSA P036D MRC-Holland, Amsterdam, The Netherlands (<http://www.mrc-holland.com>). Each subtelomeric rearrangement was identified by at least one additional MLPA analysis using the SALSA P070 as the first level screening. Afterwards, SALSA P036D was utilized for confirmatory analysis. Fluorescence *in situ* Hybridization (FISH) analysis was performed using commercially available probes (Vysis, Inc., Downers Grove, Illinois, USA) according to the manufacturer's recommendations. SNP array analyses were performed using the Affymetrix NsPl 250K SNP array platform (www.affymetrix.com). Copy number estimates were determined using the Copy Number Analyzer for Affymetrix Genechip Mapping (CNAG) software package version 2.

Molecular analyses were performed in subgroups of ID individuals suspected of certain genetic abnormalities. There were 13 individuals with clinical suspicion of Prader-Willi/Angelman syndrome (PW/AS), in whom molecular analyses were performed using MLPA. The SALSA P028 methylation specific probe-kit was utilized, which contains probes in the PW/AS region. Two out of these 13 individuals were strongly suspected with AS, and therefore additional direct sequencing of *UBE3A* (Angelman syndrome) was performed. In 4 individuals suspected with other specific syndromes, direct sequencing of the genes *UPF3B* and *MED12* (Lujan-Fryns syndrome), *PTEN* (Cowden syndrome), *TCF4* (Pitt-Hopkins syndrome) and *ZEB2* (Mowat-Wilson syndrome) was carried out.

In our cohort, there were 31 ID individuals with epilepsy. Direct sequencing of the epilepsy genes *SCN1A*, *LGII*, and *STXBP1* was performed. Since the *ARX* gene is X-Linked, screening was performed only in 19 male individuals.

There are 58 ID individuals in our cohort with head circumference/occipital frontal circumference (OFC) abnormalities, including 48 with microcephaly and 10 with macrocephaly. For the most prevalent microcephaly genes, *ASPM* and *WDR62*, direct sequencing was performed in all samples ($n=48$). Some microcephaly genes such as *MCPH1*, *CDK5RAP2*, *CENPJ*, *STIL* are considered to have a lower prevalence, thus direct sequencing was conducted only in 20 individual samples with the lowest OFC (below -4 SD). In the 10 individuals with macrocephaly, direct sequencing for *PTEN* gene was performed.

1.2.4. Ethical consideration

Informed consents were obtained from parents or legal representatives, and the study has been approved by the Ethical Board of the University of Diponegoro/Kariadi Hospital, Semarang, Indonesia. Abnormal test results were discussed with parents or legal representatives genetic counselling was provided.

1.3. Outline of the thesis

At the onset of the work described in this thesis, only a partial genetic diagnostic work-up of the ID population in Indonesia was performed (Hussein, 1998; Faradz et al., 1999). The first screening study for ID in a large cohort of Indonesian individuals was carried out using conventional cytogenetics and *FMR1* gene analysis with the main focus on males (Hussein, 1998; Faradz et al., 1999). Since then, genetic diagnostic work-up studies were performed only in relatively small ID populations focusing on FXS and chromosomal abnormalities. In addition, genetic testing is not a routine diagnostic tool for ID patients in Indonesia although some basic techniques such as cytogenetic analysis and PCR-based *FMR-1* gene analysis are available. Furthermore, due to the perception that the main health problems of childhood morbidity and mortality are socioeconomic and environmental such as malnutrition and infection, genetic disorders have not received much attention from the government and medical practitioners. Therefore, this study aimed at recording aetiological diagnosis of ID, performing phenotype-genotype correlation studies, and defining a diagnostic protocol for ID which is applicable in the Indonesian context. Furthermore, it was intended to strengthen the position of medical genetics in Indonesia for the benefit of patient care, teaching and research and to enhance genetic awareness of the Indonesian medical profession and the public at large, by providing evidence of an important genetic contribution into the causation of ID. In addition, the identification of the aetiology of ID is of major importance for patients and their families, since it allows reliable genetic counselling to their families and a better clinical management of the patients.

The general goal of the research described in this thesis was to obtain a better understanding of the genetic aetiology of ID in Indonesia by aiming at the following objectives:

- I. Determination of the frequency of cytogenetic abnormalities in Indonesian ID population.
- II. Determination of the frequency of FXS in Indonesian ID population.
- III. Determination of the frequency of subtelomeric deletions and duplications in Indonesian ID population.
- IV. Determination of the aetiology in subgroups of ID individuals suspected of having a specific syndrome.
- V. The establishment of diagnostic protocol for the Indonesian setting

Using conventional cytogenetic analysis with confirmation by some stand-alone molecular tests, we determined the frequency of cytogenetic abnormalities in a cohort of Indonesian individuals with ID (**Chapter 2**). The molecular identification of

FXS syndrome in Indonesian ID population allows tracking of individual's relatives with the same disorder. We determined the frequency of FXS in males and females, and documented the relatives with similar disorder (**Chapter 3**). Using MLPA, we determined the frequency of subtelomeric deletions and duplications in a cohort of Indonesian individuals with ID, who had a normal karyotype and who were FXS negative (**Chapter 4**). A subset of ID individuals was suspected of having a specific syndrome, and molecular testing of the genetic defect involved in these syndromes was carried out in these patients. Patients suspected for Prader-Willi syndrome and Angelman syndrome were tested by MLPA analysis, two individuals highly suspicious of Angelman syndrome were tested for mutations in the *UBE3A* gene. Other syndromes tested are Lujan-Fryns syndrome (*UPF3B* and *MED12*), Cowden syndrome (*PTEN*), Pitt-Hopkins syndrome (*TCF4*) and Mowat-Wilson syndrome (*ZEB2*). In larger patient cohorts molecular screening of some genes associated with ID and epilepsy (*SCN1A*, *LGI1*, *ARX*, *STXBP1* and *LGI1*), macrocephaly (*PTEN*) and microcephaly (*ASPM*, *WDR62*, *MCPH1*, *CDK5RAP2*, *CENPJ*, *STIL*), were tested. Almost all tests for these specific syndromes turned out to be negative. The positive case with Mowat-Wilson syndrome, and the results of testing the microcephaly genes are described in **chapter 5**. In **chapter 6**, the implication of this work including the establishment of a diagnostic protocol for the Indonesian setting is discussed. In addition, a general discussion and future directions towards a better genetic diagnostic workup in Indonesia are also provided.

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2

Chapter 2.

Cytogenetic abnormalities in Indonesian ID population

2.1. A cytogenetic study in a large population of intellectually disabled Indonesians (Genet Test Mol Biomarkers 2012; 16:412-7)

2.1

A cytogenetic study in a large population of intellectually disabled Indonesian

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Abstract

Genetic factors play a significant role in the etiology of intellectual disability (ID). The goal of this study was to identify microscopically visible chromosomal abnormalities in an Indonesian ID population and to determine their frequency, pattern and clinical features. A total of 527 intellectually disabled individuals from special schools and institutions in 4 different areas on Java Island, Indonesia, were screened for cytogenetic abnormalities. Additional analyses were carried out for verification or further characterization by using fluorescence *in situ* hybridization, multiplex ligation-dependent probe amplification or analysis of the *FMR1* promoter CGG(n) repeat. Of the 527 individuals with ID, chromosomal abnormalities were found in 87 cases (16.5%). Trisomy 21 was the major chromosomal abnormality, identified in 74 patients (14%). Other chromosome abnormalities included 8 X-chromosomal and 5 autosomal aberrations. Details on chromosome aberrations and confirmation analyses are discussed. This study shows that chromosomal abnormalities are an important cause of ID in Indonesia. Cytogenetic analysis is important for an adequate diagnosis in patients and subsequent genetic counseling for their families, especially in developing countries with limited facilities such as Indonesia.

Keywords: Cytogenetic abnormalities, Indonesia, intellectual disability

Introduction

Intellectual disability (ID) is a major health problem worldwide. In addition to health problems individuals with ID need more educational and psychological attention. Moreover, most of those with severe ID require lifelong nursing, guidance and surveillance (Schalock et al., 2007).

Known causes of ID are biochemical/metabolic defects, chromosomal abnormalities, mutations in single genes (Mendelian disorders and mitochondrial disorders), multi-factorial disorders with a polygenic predisposition and non-genetic causes (Chiurazzi and Oostra, 2000; van Karnebeek et al., 2005). Pathogenic chromosomal abnormalities are the most common genetic cause of ID (Stevenson et al., 2003; Mefford, 2009). Microscopically visible numerical and structural abnormalities account for 7 – 56% of cases depending on techniques used and patient selection (Fryns et al., 1986; Dereymaeker et al., 1988; Fryns et al., 1990; Felix et al., 1998; Santos et al., 2000; Shiue et al., 2004; Dayakar et al., 2010). Down syndrome is the most common chromosomal abnormality causing ID which can be easily detected by using routine chromosomal analysis (Tolmie and MacFayden, 2007).

To date, there are few data on incidence and cause of ID in Indonesia, even though, approximately 66,500 pupils have been registered in special schools for intellectually disabled individuals (Kemendiknas, 2010). This number, however, is far lower than the total number of ID individuals in Indonesia.

Cytogenetic analysis has not been recognized as a routine diagnostic tool for ID individuals in Indonesia, although the technique is available. Furthermore, genetic disorders have not received much attention from the government and medical practitioners, partly because the main health problems for childhood morbidity and mortality are socioeconomic and environmental such as malnutrition and infection.

Previous studies in the Indonesian ID population primarily focused on the Fragile X syndrome (Hussein, 1998; Faradz et al., 1999). Therefore, this study aimed to determine the prevalence and pattern of microscopically visible chromosomal abnormalities and the clinical features of positive cases in ID individuals in Indonesia.

Materials and methods

Patient selection and setting

A total of 527 participants (329 males and 198 females) were included in the study, their ages ranged from 6 to 25 years and they were from four different places on Java Island, Indonesia (Semarang, Temanggung, Yogyakarta, and Bandung). Of the 527 patients, 156 were institutionalized and 371 attended special schools. The majority of the individuals ($n=345$) appeared to be mildly intellectually disabled, 161 were

moderately disabled, and 21 were severely disabled, respectively (Table 1). Informed consent was obtained from the parents or legal representatives and the study was approved by the Ethical Board of the University of Diponegoro/Kariadi Hospital Semarang, Indonesia. All participants underwent a standardized clinical examination before blood was drawn. This examination comprised physical measurements and dysmorphological assessment.

Table 1. Characteristics of the study population

Characteristic	Participants (n)
Sex	
Male	329
Female	198
Area/city	
Semarang	327
Temanggung	134
Yogyakarta	11
Bandung	55
School type	
Institution	156
Special school	371
ID severity	
Mild	345
Moderate	161
Severe	21

ID, intellectual disability.

Peripheral blood samples were collected from December 2006 to November 2008, and cytogenetic analysis was performed on all 527 samples. Structural abnormalities were confirmed by multiplex ligation-dependent probe amplification (MLPA) or fluorescence *in-situ* hybridization (FISH). Southern blot analysis was carried out to confirm the presence of cytogenetically visible fragile sites on the X chromosome.

Chromosome cultures and preparations were carried out as described elsewhere (Blennow, 2005). One hundred metaphases were screened for fragile sites on each sample. Subsequently, chromosome analysis was performed using G-banding technique on the level of 400 – 600 bands. At least 20 metaphases were scored for each patient and karyotyped. If a mosaicism was suspected, 50 – 100 cells were counted.

FISH analysis was performed using commercially available probes (Vysis, Inc., Downers Grove, Illinois, USA) according to standard protocols as previously described

(de Bruijn et al., 2001). Genomic DNA of each patient was isolated using the salting out method (Miller et al., 1988). MLPA analysis was performed as described elsewhere (Schouten et al., 2002; Koolen et al., 2004). Several probe-kits from MRC-Holland, Amsterdam, The Netherlands, were used in these experiments: SALSA P036D and SALSA P070 (probes specifically designed for subtelomeric chromosomal imbalances), SALSA P096 (probes for several ID syndromes), and SALSA P028 (methylation specific probes for chromosome 15).

Southern blot analysis of the *FMR1* CGG(n) repeat was performed as described previously (Oostra et al., 1993; Smits et al., 1994).

Results

Chromosomal abnormalities were found in 87 (16.5%) of the 527 intellectually disabled individuals, trisomy 21 was the major chromosomal abnormality, occurring in 74 cases (14%). The latter cases consisted of 71 with a full blown classical trisomy 21 (43 males and 28 females), 2 with a mosaicism of trisomy 21 [47,XX,+21(73)/46,XX(27) and 47,XY+21(65)/46,XY(35)] respectively (Table 2), and 1 with a Robertsonian translocation (46,XX,der(14;21)(q10;q10),+21) (Table 3, Case 1). The latter patient's mother's karyotype was normal, and her father's sample was not available. Therefore, we could not determine whether this translocation was *de novo* or inherited from her father.

Table 2. Numerical chromosome aberrations detected in 527 intellectually disabled Indonesian individuals

Chromosomal abnormality	Karyotype	Cases (n)
Down syndrome	47,XX, + 21	28
	47,XY, + 21	43
	47,XX, + 21(73)/46,XX(27)	1
	47,XY, + 21(65)/46,XY(35)	1
Turner syndrome	45,X(10)/46,XX(90)	1
Other X aneuploidy	47,XXX	1

In 13 cases, chromosomal abnormalities other than Down syndrome were detected. Two participants had X-chromosomal aneuploidies (45,X(10)/46,XX(90) and 47,XXX; Table 2). For both females the chromosomal aberration detected is not a satisfactory explanation for their moderate ID. The other 11 cases are structural chromosome aberrations (cases 2-12, Table 3).

Table 3. Structural chromosome aberrations detected in 527 intellectually disabled Indonesian individuals

Case no.	Karyotype	Molecular confirmation	Parents
1	46,XX,der(14;21)(q10;q10), + 21	NT	Maternal karyotype normal; paternal karyotype unavailable
2	46,XX,del(18)(q21.3-qter)dn	FISH: Del 18qter	Normal karyotypes
3	46,XY,der(4)t(4:8)(p16;p23)dn	FISH and MLPA: Del4pter/dup8pter	Normal karyotypes
4	46,XX,der(10)t(4:10)(p16;q26)	FISH and MLPA: Del10q/dup4p	NT
5	46,XX t(3;12)(p14.1;q21.2)	MLPA: Normal	NT
6	47,XY,idic(15)(q13)	MLPA: Dup 15 (maternal origin)	NT
7	46,XX,del(X)(q21-qter)	NT	NT
8	46,XY, fra(X)(q27.3)	SB, full mutation	Mother is premutation carrier
9	46,XX, fra(X)(q27.3)	SB, full mutation	Mother is premutation carrier
10	46,XY, fra(X)(q27.3)	SB, full mutation	Mother is premutation carrier
11	46,XY, fra(X)(q27.3)	SB, premutation–full mutation (mosaic)	Mother is premutation carrier
12	46,XY, fra(X)(q27.3)	SB, premutation–full mutation (mosaic)	Mother is premutation carrier

FISH, fluorescent *in situ* hybridization; MLPA, multiplex ligation-dependent probe amplification; NT, not tested; SB =Southern blot analysis.

Apart from the t(14;21) case, autosomal structural abnormalities were found in 5 cases (1.0%): 2 unbalanced translocations, 1 balanced translocation, 1 deletion, and 1 isodicentric chromosome. No further confirmation test was performed on the Down syndrome cases, the cases with an X-chromosomal aneuploidy (Table 2) and a case with the large visible terminal Xq deletion (case 7). Five samples from 4 males and 1 female patient were identified to have a fragile site at Xq27.3 (cases 8-12). Southern blot analysis confirmed the presence of a fully methylated expansion (>200 CGG repeats) in the promoter region of the *FMR1* gene in each of the 5 cases.

MLPA or FISH analysis was used to confirm the structural chromosomal abnormalities in cases with autosomal aberrations (cases 2-6). Whole chromosome paints of chromosome 18 confirmed a missing part of chromosome 18 in the sample of the patient with 46,XX,del(18)(q21.3→qter)dn (case 2). Further analysis using an 18q telomere FISH probe detected only 1 signal from chromosome 18. Her parent's karyotypes were normal, confirming *de novo* occurrence. This patient had clinical features resembling those of previously described patients with a similar chromosomal aberration (Kimpen et al., 1991; Kline et al., 1993) (Figure 1).

Figure 1. Patients with a chromosomal abnormality

(A) Case 2. (B) Case 3. (C) Case 4. (D) Case 5. (E) Case 6. (F) Case 7.

In case 3, cytogenetic analysis revealed 46,XY,del(4)(p16). However, confirmation with MLPA analysis demonstrated not only a deletion of chromosome 4pter but also a duplication of 8pter (SALSA MLPA kits P036D and P070, MRC-Holland, Amsterdam, The Netherlands). Afterwards FISH was performed by using probes for the subtelomeric regions of chromosome 4p and 8p indeed only 1 signal for 4pter and 3 signals for 8pter (1 of which was on the derivative chromosome 4) were detected. Both parents showed normal karyotypes, and carriership of a balanced translocation has been excluded. Therefore, the karyotype of the patient should be designated as: 46,XY,der(4)t(4:8)(p16;p23)dn. Further characterization was performed with MLPA analysis by using several probes from the Wolf-Hirschhorn Syndrome Critical Region (WHSCR) (SALSA MLPA kit P096). This analysis revealed a deletion of the entire WHSCR. The cytogenetic and molecular analyses confirmed the clinical diagnosis of Wolf-Hirschhorn syndrome (Figure 1).

In case number 4 cytogenetic analysis revealed a karyotype of 46,XX,add(10)(q26). However, MLPA demonstrated a deletion of chromosome 10qter and a duplication of 4pter. Subsequently, FISH was performed using probes for the subtelomeric regions of chromosome 10q and 4p. Only 1 signal for 10qter and 3 signals for 4pter (1 of which on the aberrant chromosome 10q) were detected. Consequently,

the karyotype of the patient should be designated as: 46,XX,der(10)t(4:10)(p16;q26). Unfortunately, this patient's parents were unavailable for testing. Because 4pter duplications are reported in patients with and without ID and distinctive facial features (Gerard-Blanluet et al., 2004; Rodriguez et al., 2007), it is suggested that the phenotype in case 4 most likely is due to the deletion of 10qter. The clinical features of this patient are in concordance with the consistent phenotype of patients with a 10q26.1qter deletion, as described by (de Vries et al., 2003) (Figure. 1).

In case 5 (Figure. 1), in which chromosome analysis revealed a 46,XX,t(3;12)(p14.1;q21.2) karyotype, further confirmation using MLPA showed a normal result. It is therefore suggested that the aberration was a (cytogenetically) balanced translocation. Neither parent was unavailable for testing. In case 6 cytogenetic analysis showed a 47,XY,idic(15)(q13). MLPA analysis of probes in the 15q11.2-15q15.1 region (MRC Holland kit P028) showed 4 copies of the probes between BP1 and BP4 (including *TUBGCP5* and *TJP1*), and 3 copies of the probes in the *TRPM1*, *KLF13*, and *CHRNA7* genes (between BP4 and BP5) (Miller et al., 2009). The methylation specific analysis indicated that the marker was of maternal origin. The patient's parents were unavailable for testing. Clinical features were severe ID, epilepsy and very poor language expression, which are in fact the main features of idic (15) syndrome (Battaglia, 2008) (Figure. 1).

Case 7 had a deletion of part of the long arm of one of her X chromosomes [46,X,del(X)(q21→qter)]. She had mild ID and obvious dysmorphisms (Fig. 1). Females with a similar aberration have been reported to show mostly only mild Turner stigmata or subtle dysmorphisms, next to ovarian failure. ID, however, was not reported in these females (Graham et al., 2007), which makes it unlikely that the chromosomal aberration directly causes the ID in this patient and further investigations such as X inactivation studies are needed.

Four males and 1 female expressed a fragile site on the X chromosome. Southern blot analysis confirmed that they all had fragile X syndrome. Three cases (patients 8, 9 and 10) had a full mutation of *FMR1*, and 2 (cases 11,12) showed a mosaicism (premutation and full mutation).

After exclusion of individuals with chromosomal aberrations other than Fragile-X syndrome (82 patients for the whole population and 46 for the male population), the prevalence of Fragile-X syndrome in this study is 1.1% (5 of 445) among the whole study population and 1.4% (4 of 283) in the male population.

Discussion

The overall frequency of microscopically visible chromosomal aberrations in this study was 16.5%. This is similar to the rate reported in other studies (13.3%-17.6%) (Fryns et al., 1986; Dereymaeker et al., 1988; Fryns et al., 1990), although different frequencies were found in other studies: 7.9% (van Karnebeek et al., 2002), 22.4% (Shiue et al., 2004), 28.6% (Santos et al., 2000), 34.2% (Felix et al., 1998) and 56% (Dayakar et al., 2010). These differences might be due to variations in inclusion criteria of patients. van Karnebeek and colleagues found a lower frequency of microscopically visible aberrations, possibly because the study was performed in a tertiary care centre (outpatient clinic) (van Karnebeek et al., 2002). Some studies generated higher frequencies than our study (Table 4) and this may have occurred because more patients with moderate and severe or profound ID were included. These differences might also be due to pre-selection of cases without known non-chromosomal causes of ID or multiple congenital anomalies, as was done by Dayakar et al., (2010).

Table 4. Frequency of microscopically visible chromosomal aberrations in current study compared with previous studies

Study (year)	Overall frequency	Structural abnormalities	Numeric abnormalities	Down syndrome in patients with cytogenetic abnormalities
van Karnebeek et al. (2002)	7.9 (21/266)	4.9 (13/266)	3.0 (8/266)	0 (0/21)
Dereymaeker et al. (1988)	13.3 (21/158)	3.8 (6/158)	9.5 (15/158)	71.4 (15/21)
Fryns et al. (1986)	15.0 (26/173)	2.3 (4/173)	12.7 (22/173)	84.6 (22/26)
Current study	16.5 (87/527)	2.3 (12/527)	14.2 (75/527)	85.1 (74/87)
Fryns et al. (1990)	17.6 (46/262)	1.2 (3/262)	16.4 (43/262)	93.4 (43/46)
Shiue et al. (2004)	22.4 (94/419)	2.6 (11/419)	19.8 (83/419)	81.9 (77/94)
Santos et al. (2000)	28.6 (28/98)	6.1 (6/98)	22.5 (22/98)	42.9 (12/28)
Felix et al. (1998)	34.2 (69/202)	1.5 (3/202)	32.7 (66/202)	94.2 (65/69)
Dayakar et al. (2010)	56 (56/100)	11 (11/100)	45 (45/100)	51.78 (29/56)

Data are expressed as % (n/n).

The-male-to-female ratio in our study was 1.66:1 which is higher than in some previous publications (1.2:1 - 1.4:1) (Roeleveld et al., 1997; Partington et al., 2000; Macayran et al., 2006; Lin, 2009), but lower than in other studies, which reported a male to female ratio as high as 3:1 (Shin and il, 1999; Tang et al., 2008). The sex ratio differences might be explained by the differences in selection and case ascertainment (Roeleveld et al., 1997). Another ascertainment bias in our setting might be that parents seek assistance more frequently for boys than for girls, due to generally higher expectation for male children.

Fourteen percent of the intellectually disabled individuals in this study had Down syndrome. This finding confirms that the syndrome is the most common chromosomal abnormality involved in ID. Severe cases, such as trisomy 13 and trisomy 18 were not found in our study, most likely because these patients died before they reached school age. The prevalence of Down syndrome in our study is similar to that in previous studies conducted in the Indonesian population (12–14%) (Hussein, 1998). In addition, the prevalence of Down syndrome in our study resembled the frequency of 13 – 15% reported in a white population (Matilainen et al., 1995; van Buggenhout et al., 1999). We found a male-to-female ratio of 1.5:1 in Down syndrome cases, which reflects the male excess in our study population. The proportion of patients with Down syndrome among all male (43 of 329 [13%]) and all female (28 of 198 [14%]), participants, however, was similar, which corresponds to previous reports in this population (Hussein, 1998).

The prevalence for Fragile-X syndrome in this study was 1.1% (5 of 445) among the whole study population and 1.4% (4 of 283) in the male population. A previous study among intellectually disabled individuals in Indonesia that used molecular analysis, showed a similar FXS prevalence of 1.9% (5 of 262) in the male population (Faradz et al., 1999). It is also similar to that reported in some studies of white populations (2-3%) (de Vries et al., 1997; Hecimovic et al., 2002; Biancalana et al., 2004). The prevalence could have been higher if molecular analysis had been performed in all 527 patient samples, because not all carriers of the FRAXA mutation express the fragile site on karyotyping (Pembrey et al., 2001). However, because of limited availability of molecular testing in Indonesia, cytogenetic studies for fragile-X are still a useful tool to detect fragile-X(A) and other fragile site abnormalities, including *FRAXE*, *FRAXF* and fragile sites in autosomes (Hussein, 1998).

Our study shows that cytogenetic analysis is still a powerful tool to detect genetic abnormalities in the ID population. The fact that cytogenetic analysis can now be performed in Indonesia should be considered by granting agents, such as government and non-profit organizations, so that they may financially support genetic studies in developing countries such as Indonesia. Furthermore, because common infectious diseases and nutritional problems are becoming less prevalent in Indonesia, diagnostic facilities for genetic diseases must receive a higher priority. Such efforts would extend genetic analysis to more diverse populations than normally studied (Bustamante et al., 2011).

Conclusions

Chromosomal abnormalities play an important causative role in ID in Indonesia. However, because cytogenetic analysis is still not commonly performed in intellectually disabled individuals in Indonesia, the implementation of this technique in a routine diagnostic setting will help to establish a genetic diagnosis in individuals with ID in the local setting, and will improve the possibilities for genetic counseling to the families.

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Disclosure statement

No competing financial interests exist.

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3

Chapter 3.

Fragile-X syndrome in Indonesian ID population

3.1. Prevalence of Fragile X syndrome in males and females in Indonesia (World J Med Genet 2012; 2:15-22)

Prevalence of Fragile X syndrome in males and females in Indonesia

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Abstract

Aim: To investigate the prevalence of Fragile X syndrome (FXS) in intellectually disabled male and female Indonesians.

Methods: This research is an extension of a previously reported study on the identification of chromosomal aberrations in a large cohort of 527 Indonesians with intellectual disability (ID). In this previous study, 87 patients had a chromosomal abnormality, five of whom expressed fragile sites on Xq27.3. Since FXS cannot always be identified by cytogenetic analysis, molecular testing of the fragile X mental retardation 1 CGG repeat was performed in 440 samples. The testing was also conducted in the five previously identified samples to confirm the abnormality. In total, molecular study was conducted in 445 samples (162 females and 283 males).

Results: In the cohort of Indonesian ID population, the prevalence of FXS is 9/527 (1.7%). The prevalence in males and females is 1.5% (5/329) and 2% (4/198), respectively. Segregation analysis in the families and X-inactivation studies were performed. We performed the first comprehensive genetic survey of a representative sample of male and female ID individuals from institutions and special schools in Indonesia. Our findings show that a comprehensive study of FXS can be performed in a developing country like Indonesia where diagnostic facilities are limited.

Conclusion: The prevalence of FXS is equal in females and males in our study, which suggests that the prevalence of FXS in females could be underestimated.

Key words: Fragile X syndrome (FXS); Intellectual Disability (ID); Fragile X Mental Retardation 1; CGG Repeat; Indonesia

Introduction

Fragile X syndrome (FXS) is the most common form of inherited intellectual disability (ID), with an estimated prevalence of 1 in 4000-6000 males and 1 in 7000-10 000 females (Crawford et al., 2001). Expansion of a CGG repeat in the 5' untranslated region of fragile X mental retardation 1 (*FMR1*) is the most frequent cause of FXS (Strom et al., 2007; Hill et al., 2010). When the expansion exceeds the number of 200 repeats (full mutation), the promoter region becomes hypermethylated and the *FMR1* gene is silenced. This leads to deficiency of the FMR1 protein (FMRP) (Chiurazzi et al., 2004). FXS is inherited as an X-linked dominant disease with variable expressivity and reduced penetrance in females. The level of ID in FXS males ranges from mild to profound, whilst females are usually less affected (Hagerman and Hagerman, 2002; Oostra and Willemsen, 2002).

Several behavioral characteristics associated with FXS include autism spectrum disorders, poor eye contact, short attention span, hyperactivity, several stereotypic behaviors (hand flapping, hand biting, preservative speech, echolalia), tactile defensiveness and anxiety related to social contact (Hagerman et al., 1992; Symons et al., 2003; Kaufmann et al., 2004; Budimirovic et al., 2006). The classical facial phenotype of FXS includes a prominent forehead, a long, narrow face, a prominent jaw, and prominent ears. The palate is often highly arched. Macro-orchidism is reported in more than 80% of post-pubertal and adult males. Connective tissue abnormalities such as soft velvet-like skin, joint hypermobility, pes planus, congenital hip dislocation, scoliosis and clubfoot are also commonly observed (Hagerman and Hagerman, 2002; Hersh and Saul, 2011).

Diagnostic analysis of FXS is mainly based on direct amplification of the CGG-repeat using flanking primers and Southern blot analysis (Fu et al., 1991; Oostra et al., 1993; Smits et al., 1994; Zhou et al., 2004; Strom et al., 2007; Hantash et al., 2010). Standard PCR testing allows amplification of alleles up to 120-150 CGGs. Although this method cannot reveal full mutations, it allows precise sizing of premutation alleles. On the contrary, Southern blot analysis allows sizing of full mutations but is unable to discriminate between large normal and small premutation alleles (Zhou et al., 2004). To overcome these problems, several diagnostic laboratories recently changed their procedure to PCR-based tests that can amplify repeat alleles up to full mutations and are able to distinguish between female samples homozygous for a normal allele or heterozygous for a normal and an expanded allele (e.g., tests by Abbott, IL, United States, and Asuragen Inc, Austin, United States). While these procedures are routinely performed in the Western world, they are not being used as standard diagnostic tools in Indonesia, mainly due to costs and lack of adequate health insurance coverage.

In a previous study, the prevalence of FXS in the male Indonesian population was determined to be 1.9% (5/262) (Faradz et al., 1999). However, diagnostic testing for FXS is not routinely performed and widely available in Indonesia. Therefore, we aimed to identify unrecognized FXS individuals and to determine the prevalence in both male and female individuals with ID. In view of the fact that genetic testing is still uncommon practice in Indonesia, the detection of new FXS cases gives insight in to the prevalence of FXS in Indonesia and should promote awareness of this disease among medical doctors and health professionals in Indonesia. For the families involved, establishing a diagnosis will be beneficial since genetic counseling and carrier testing can be provided.

Materials and methods

Selection and setting

This research is an extension of a previously reported study on the identification of chromosomal aberrations in a large cohort of 527 Indonesian ID patients from several special schools and institutions in Java Island, Indonesia. In this previous study, 87 patients had a chromosomal abnormality, five of whom expressed fragile sites on Xq27.3 (Mundhofir et al., 2012). Since FXS cannot always be identified by cytogenetic analysis, molecular testing of the *FMR1* CGG repeat was performed in 440 samples. The testing was also conducted in five previously identified samples to confirm the abnormality. In total, molecular study was conducted in 445 samples (162 females and 283 males).

Genomic DNA of each patient was isolated using the salting out method as described elsewhere (Miller et al., 1988), with slight modification. The CGG repeat in the *FMR1* promoter was amplified as described by Fu et al., (1991). Fragment length analysis was carried out on an ABI Prism 3730 DNA Analyzer (Life Technologies, Foster City, United States) and the Genemapper software (version 4.0, Apache) was used to determine the exact length of the CGG repeat. Southern blot analysis of the *FMR1* CGG(n) repeat was performed as described previously. In families 5, 6 and 7 (Figure 1), a more detailed analysis of the repeat length was performed using a three-primer CGG repeat primed *FMR1* PCR method (Asuragen Inc, Austin, USA), according to the manufacturer's protocol. The difference between the distribution of the full mutation allele in males and females was calculated using a χ^2 test.

A clinical reinvestigation was done in the positive cases and family members at risk of being a carrier were molecularly tested. X-chromosome inactivation (XCI) analyses were performed in all full mutation females in order to explain their phenotypes. Family members from all affected individuals were counseled and

extended pedigrees were drawn. Thirty nine family members were available for molecular testing and clinical examination was only performed in family members with obvious signs of ID. The XCI pattern was studied in female samples with a full mutation (either clinically affected or unaffected) as described before (Spath et al., 2010).

Results

In a total of 445 (162 females and 283 males) molecularly tested individuals (607 alleles) 593 alleles are within the normal range (15-44 CGG repeats), 3 alleles in the intermediate range (45-55 CGG repeats), 2 alleles in the premutation range (between 55 and 200 CGG repeats) and 9 alleles in the full mutation range (> 200 CGG repeats) (classification according to the American College of Obstetricians and Gynecologists Committee Opinion No. 469). The 29 allele ($n=245$) was the most frequent allele in this population, followed by 28 CGG repeats ($n=127$) and 30 CGG repeats ($n=93$).

The five samples (4 males and 1 female) in which fragile sites were shown in previous chromosome analyses indeed showed a full mutation with Southern blot analysis, therefore confirming the diagnosis of FXS. Another four samples (1 male and 3 females) were newly identified to have a full mutation. Two of the positive male samples showed a mosaic pattern of premutation to full mutation (Family 2/III:2 and Family 5/III:2, Table 1). A χ^2 test revealed no statistically significant differences in the distribution of full mutation alleles between males and females ($\chi^2 = 0,184$; $df = 1$; $P = 0.67$).

Table 1. Fragile X mental retardation 1 gene analysis of index fragile X syndrome subjects

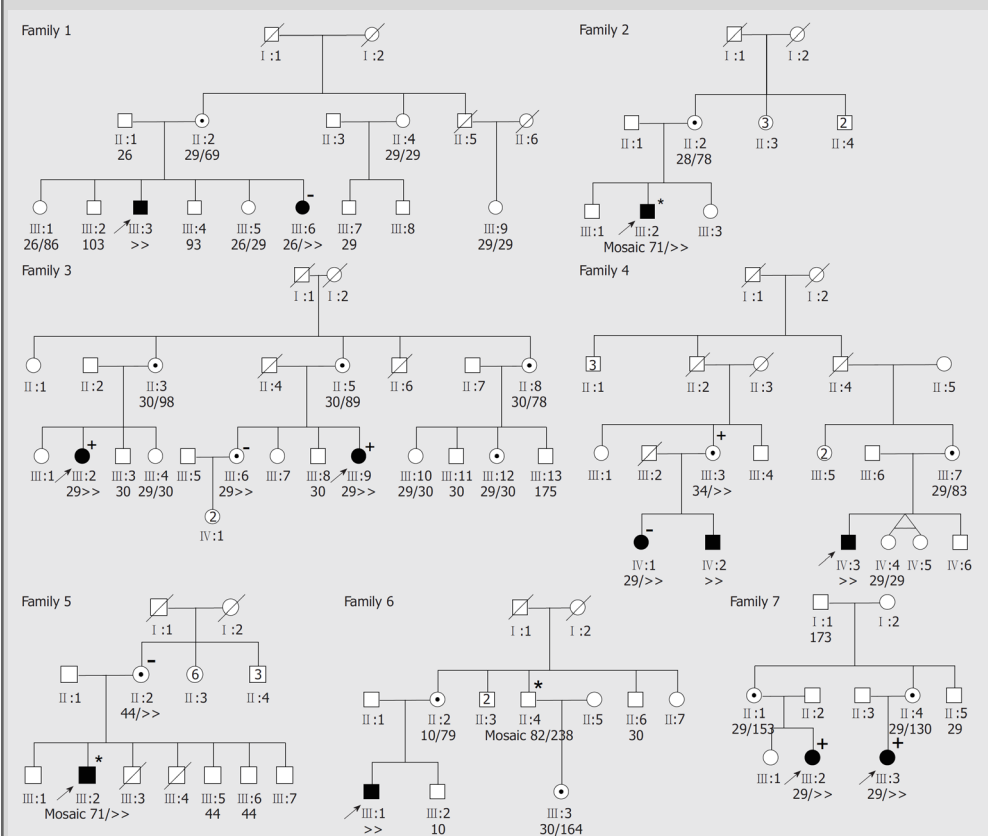
Patient	Sex	FMR1 gene analysis
Family1/III:3	Male	Full mutation
Family 2/III:2	Male	Mosaic premutation/full mutation
Family 3/III:2	Female	Full mutation
Family 3/III:9	Female	Full mutation
Family 4/IV:3	Male	Full mutation
Family 5/III:2	Male	Mosaic premutation/ full mutation
Family 6/III:1	Male	Full mutation
Family 7/III:2	Female	Full mutation
Family 7/III:3	Female	Full mutation

FMR1: Fragile X mental retardation 1.

Pedigree analysis of the nine FXS cases showed that two individuals were related to two other index cases in other families (first cousins in two different families)

(Family 3/III:2 and III:9; Family 7/III:2 and III:3, Table 1). Therefore, only seven families were identified in this study (Figure 1). Molecular testing of potential carriers in those families resulted in the identification of 17 samples with a premutation (11 females and 6 males). In one of the 11 females (Family 6/III:3), the Southern blot result could not clearly distinguish between a large premutation or full mutation. More detailed analysis using repeat-primed PCR (Asuragen Inc., Austin, United States) revealed a premutation. Furthermore, another six full mutation cases were identified: five females (two mildly affected and three clinically unaffected) and one clinically affected male.

Figure 1. Pedigrees of Fragile X syndrome families



Besides the nine index cases (indicated by an arrow), six additional family members with a full mutation (five females, full black circle and one male, indicated by full black square) and 17 individuals [11 males; 5 females (indicated by dotted circle)] with a premutation have been identified. The length of the CGG repeats is depicted below the pedigree number of each tested individual. The X-inactivation (XCI) of full expansion females is shown at the upper right of the pedigree symbol (+ for non-random XCI and - for random XCI). Asterisk at the upper right indicated a mosaic premutation to full mutation.

X-inactivation studies were performed in all nine females with a full mutation. Four females (two mildly affected and two unaffected) showed random patterns of inactivation (Table 2). Four out of five samples with non random patterns of inactivation [$> 80:20$, defined by Amos-Landgraf et al., (2006)] are from clinically affected females, while the 5th one is not affected. Southern blot analysis showed that in the unaffected female the normal allele was active and the expanded allele was inactive, whereas in the affected females the normal allele was inactive.

Table 2. X chromosome inactivation pattern in all full mutation females

Pedigree	XCI	Clinical features
Family 3/III:2	87/13 (non random) ¹	Affected
Family 3/III:9	96/4 (non random) ¹	Affected
Family 7/III:2	93/7 (non random) ¹	Affected
Family 7/III:3	82/18 (non random) ¹	Affected
Family 1/III:6	75/25 (random)	Affected (mild)
Family 4/IV:1	74/26 (random)	Affected (mild)
Family 3/III:6	60/40 (random)	Not affected
Family 4/III:3	84/16 (non random) ²	Not affected
Family 5/II:2	67/33 (random)	Not affected

¹The normal allele is inactive by X chromosome inactivation (XCI) and the expanded allele is active but methylated because of the expansion; ²the normal allele is active and the expanded allele is not active.

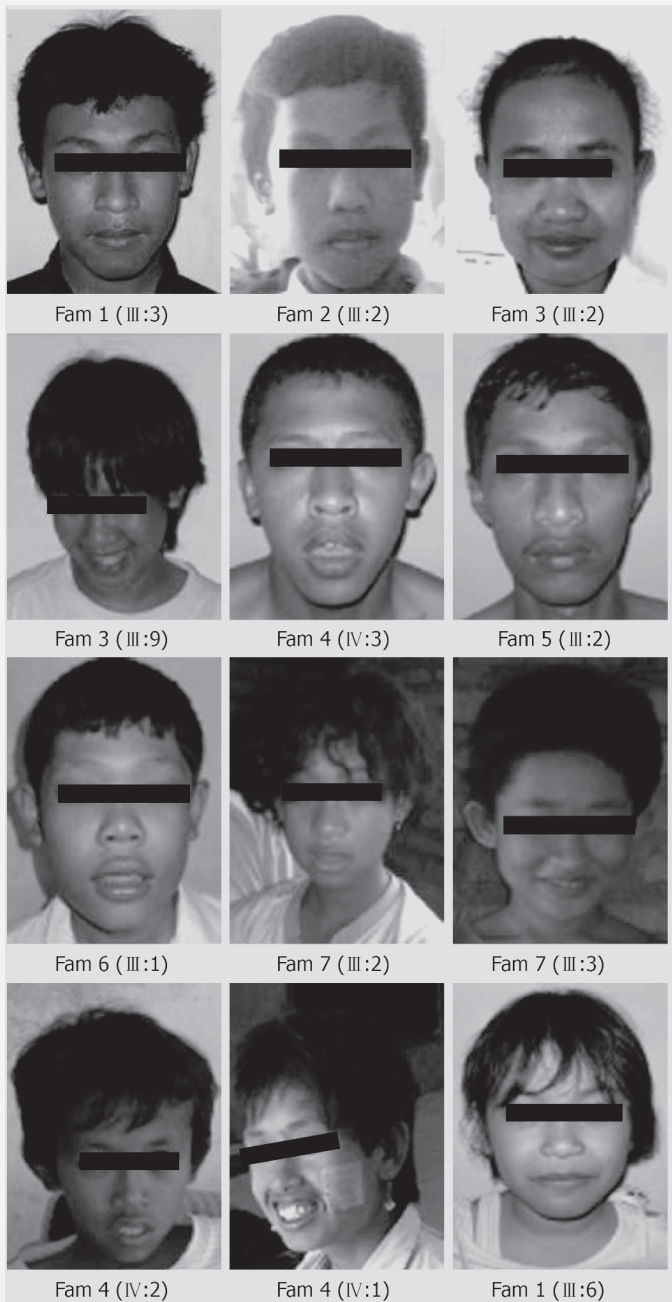
A summary of the most common features of the index patients and affected family members is shown in Table 3, for male and female individuals, respectively. Clinical pictures of index patients and affected family members are depicted in Figure 2. In male individuals, shy behavior (shy and timid behavior with a tendency towards social withdrawal) and social anxiety are the most frequent features (detected in all six males=100%), followed by large cupped ears, elongated face and joint laxity (detected in 83%). Four of post pubertal individuals had large testicular size (67%). Highly arched palate, scoliosis and flat feet were found in three patients (50%), whereas neurological problems (seizure, spasticity of the extremities and strabismus) were found in one patient. In females, shy behavior and social anxiety are the most frequent features (100%), whereas joint laxity and flat feet were found in five (83%). Four females showed large cupped ears, elongated face and high arched palate (67%). Scoliosis and strabismus were found in three and two individuals, respectively (50% and 33%).

Table 3. Clinical features of male and female patients with Fragile X syndrome

	Male						Female							
	Fam 1/ III : 3	Fam 2/ III : 2	Fam 4/ IV : 3	Fam 5/ III : 2	Fam 6/ III : 1	Fam 4/ IV : 2	%	Fam 3/ III : 2	Fam 3/ III : 9	Fam 7/ III : 2	Fam 7/ III : 3	Fam 4/ IV : 1	Fam 1/ III : 6	%
Intellectual disability level	2	1	2	2	1	2		2	2	3	2	1	1	
Shy behavior and social anxiety	+	+	+	+	+	+	100	+	+	+	+	+	+	100
Large cupped ears	+	+	+	-	+	+	83	-	+	+	+	+	-	67
Elongated face	+	+	+	+	-	+	83	-	+	+	+	+	-	67
High arched palate	+	-	+	-	+	-	50	+	+	+	-	+	-	67
Scoliosis	-	-	+	+	-	+	50	-	+	+	-	+	-	50
Joint laxity	+	+	+	+	-	+	83	-	+	+	+	+	+	83
Neurological problems	-	-	+	-	-	-	17	-	+	+	-	-	-	33
Macroorchidism	+	+	+	+	- ¹	- ¹	67							
Flat feet	+	+	+	-	-	-	50	+	+	+	+	+	-	83

¹Prepubertal. 1: Mild; 2: Moderate; 3: Severe. Fam: Family; %: Percentage.

Figure 2 Clinical pictures of patients with Fragile X syndrome.



Fam: Family.

Discussion

Few studies have been carried out to determine the contribution of FXS as a cause of ID in the Indonesian population (Faradz et al., 1999; Winarni et al., 2012). In this study, we performed a comprehensive genetic survey of a representative sample of male and female ID individuals from institutions and special schools. The prevalence of FXS found in this study was 1.7% (9/527); 1.5% (5/329) in the male population and 2% (4/198) in the female population. This prevalence of FXS is similar to that in other Asian populations (approximately 1%-3%) (Pang et al., 1999; Kwon et al., 2001; Pandey et al., 2002) and is about the same as found in a previous study from Indonesia with a prevalence of 1.9% (5/262) in the male population (Faradz et al., 1999).

The prevalence of FXS among females was estimated to be about half of that of males (Crawford et al., 2001). The actual distribution of full mutation alleles in the general population, however, is considered to be equal in both males and females (Hagerman, 2008), but due to the X-inactivation in females, they are usually less severely affected. With regards to the allele distribution of full mutation alone, this study yielded no statistically significant differences in males 1.5% (5/329) and females 2% (4/198). This finding is in line with the results of previous studies (Pang et al., 1999; Hagerman, 2008). The equal distribution of clinically affected females and males in the present study, however, was unexpected. In order to explain why most of the females with a full mutation in this study are clinically affected, the X inactivation status was determined. All female index patients ($n=4$) demonstrated a non random XCI, in which the normal unexpanded allele was preferentially inactivated. Although the XCI pattern in blood does not necessarily represent the pattern in the brain, the results in this family provide evidence for the fact that XCI patterns play a role in the development of cognitive disturbances in females with a full mutation. The results of the XCI assay in the five female family members with a full mutation are also in concordance with their clinical status: the two mildly affected females and two of the unaffected females showed random X-inactivation. The difference in intellectual abilities between these females might possibly be explained by a difference in the X-inactivation pattern in brain. One of the unaffected females showed non-random X-inactivation (Family 4/III:3), but since the normal allele was preferentially active, this explains why she has a normal phenotype. This study clearly demonstrates why females with full mutation alleles can be affected or unaffected, depending on their XCI pattern, a feature that has been recognized before (Reiss et al., 1995; de Vries et al., 1996; Heine-Suner et al., 2003; Migeon, 2006). The percentage of clinically affected females (due to non random X-inactivation) in the present study is considerably higher than what has been reported among the full mutation female population: 44% (4/9) in this study vs 24.1% (7/29)

reported by Reiss et al., (1995). Further studies on larger numbers of full mutation females have to be performed in order to confirm this high percentage of non-random X-inactivation in our female population.

In family 5, the affected index male (III:2) showed a mosaic premutation to full mutation (71/>>). Segregation analysis using Southern blot in the family demonstrated that the mother was a carrier of a full mutation. In order to exclude the possible presence of a low amount of premutation alleles in the mother, an additional test using a repeat-primed PCR was performed. The analysis confirmed that the mother was a carrier of a normal allele (44 CGG repeats) and a full mutation allele (294 CGG repeats) without evidence of mosaic premutation allele. This indicates that the full mutation allele of the mother was transmitted to her son in reduced size. Although the molecular mechanisms responsible for the reduction of the CGG repeat in the *FMR1* gene are largely unclear, several other cases where full mutation carrier females had affected sons with a mosaic pattern, have been described (Malzac et al., 1996; Rousseau et al., 1991). One of the mechanisms explaining repeat contraction (but also expansion) is slipped strand mispairing (Chiurazzi et al., 1994; Tabolacci et al., 2008). Another explanation is that the contraction could be a postzygotic event due to somatic instability of the CGG repeat (Dobkin et al., 1996; Reyniers et al., 1999; Taylor et al., 1999).

Individuals who have a mosaic premutation to full mutation may have a milder phenotype compared to those with a full mutation (Cohen et al., 1996). Besides patient III:2 from family 5, patient III:2 from family 2 also showed a mosaic pattern on the Southern blot. Notably, one of the male family members with a normal intelligence was also identified to have a mosaicism of a premutation (81 CGG repeats) and a full mutation allele (Family 6/II:4). However, the full mutation allele was not visible on the Southern blot and was only detected after the repeat-primed PCR which was performed in order to better characterize the repeat number in his daughter. This may indicate that the fully expanded allele was present only in a small percentage of cells, explaining the normal phenotype.

The most frequent clinical features found in both sexes in our population were shy behavior and social anxiety, large cupped ears, elongated face and joint laxity. These features were consistent with those described for FXS (Hagerman et al., 1992; Hagerman and Hagerman, 2002; Hersh and Saul, 2011).

Cytogenetic testing to detect FXS is no longer considered to be sufficiently accurate because of its high false negative and false positive rates (Hersh and Saul, 2011), the main difficulty being the detection of females with a full mutation (Jenkins et al., 1991; Sutherland et al., 1991). Indeed, in our study cytogenetic analysis only picked up five out of nine samples, most of which were males. Although cytogenetic diagnosis

is still useful and affordable to establish a FXS diagnosis in developing countries, this study emphasizes the significance of molecular screening. Moreover, despite the fact that the PCR-based test is available at the Center for Biomedical Research (CEBIOR) at Diponegoro University, testing for FXS in the ID population in Indonesia is not routinely performed and CEBIOR is the only laboratory to perform FXS diagnosis in Indonesia. It is recognized that FXS is an inherited disease; however, establishing a diagnosis and providing possibilities for genetic counseling and carrier testing is not seen as useful in Indonesia. Due to its high costs and limited accessibility, prenatal diagnosis is only available to a minority of the population. Even though termination of pregnancy is legal when based on a medical emergency, e.g. genetic diseases (Republic Indonesia Laws No. 36 / 2009), in practice it still is a very complex procedure. Also, other options such as preimplantation genetic diagnosis are financially and culturally complex. Still, as common infectious diseases and nutritional problems are becoming less prevalent in Indonesia, diagnostic facilities for inherited diseases such as FXS need to get a higher priority. In addition, medical personnel and stake holders at the Ministry of Health should be continuously informed about the problem of genetic diseases and its management.

FXS testing is a common diagnostic procedure performed in all non-microcephalic males with ID of unknown origin in Western countries (Ropers and Hamel, 2005). However, routine FXS testing in females with ID of unknown origin is said not to be warranted unless there are other indicators (e.g., a positive family history) (van Karnebeek et al., 2005). On the other hand, the American College of Medical Genetics strongly recommends fragile X testing to be considered in both genders with unexplained ID, especially in the presence of any physical or behavioral characteristics of FXS, a positive family history and relatives with undiagnosed ID (Sherman et al., 2005). Our findings support the notion to broaden FXS testing to include females, in view of the fact that the prevalence of FXS in females could be higher than thought up to now.

Acknowledgments

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Comments

Background

Fragile X syndrome (FXS) is the most common form of inherited intellectual disability (ID). Expansion of a CGG repeat in the 5' untranslated region of fragile X mental retardation 1 (*FMR1*) gene is the most frequent cause of FXS.

Research frontiers

Diagnostic analysis of FXS is mainly based on direct amplification of the CGG repeat using flanking primers and Southern blot analysis. While these procedures are routinely performed in the Western world, they are not being used as standard diagnostic tools in Indonesia, mainly due to costs and the lack of adequate health insurance coverage.

Innovations and breakthroughs

In the previous study, the prevalence of FXS in the male Indonesian population was determined; however, diagnostic testing for FXS is not routinely performed and not widely available in Indonesia. Therefore, the authors aimed at identifying unrecognized FXS individuals and determining the prevalence in both male and female individuals with ID. They performed the first comprehensive genetic survey of a representative sample of male and female ID individuals from institutions and special schools in Indonesia.

Applications

Their findings show that a comprehensive study of FXS can be performed in a developing country like Indonesia where diagnostic facilities are limited. Moreover, their findings support the notion to broaden FXS testing to include females, in view of the fact that the prevalence of FXS in females could be higher than thought up to now.

Terminology

FXS is the most common inherited cause of ID. The spectrum of ID ranges from mild to severe, while physical features can include an elongated face, large and prominent ears, larger testes/macroorchidism (in males), behavioral characteristics such as stereotypic movements, and social anxiety. FXS is caused by mutations in the *FMR1*-gene. *FMR1* is a gene in humans which encodes a protein called fragile X mental retardation protein. This protein is important for normal cognitive development.

Peer review

This is a good descriptive study in which the authors investigate the prevalence of FXS in intellectually disabled male and female Indonesians. The results are interesting and suggest that the prevalence of FXS in females could be underestimated.

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4

Chapter 4.

Subtelomeric deletions and duplications in Indonesian ID population

- 4.1. Subtelomeric chromosomal rearrangements in a large cohort of unexplained intellectually disabled individuals in Indonesia: A clinical and molecular study (submitted)
- 4.2. Monosomy 9pter and trisomy 9q34.11qter in two sisters due to a maternal pericentric inversion (Gene 2012; 511:451-4)

Subtelomeric chromosomal rearrangements in a large cohort of unexplained intellectually disabled individuals in Indonesia: A clinical and molecular study

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Abstract

Context: Unbalanced subtelomeric chromosomal rearrangements are often associated with intellectual disability (ID) and malformation syndromes. The prevalence of such rearrangements has been reported to be 5-9% in ID populations.

Aims: To study the prevalence of subtelomeric rearrangements in the Indonesian ID population.

Methods and Material: We tested 436 subjects with unexplained ID using multiplex ligation dependent probe amplification (MLPA) using specific designed sets of probes to detect human subtelomeric chromosomal imbalances (SALSA P070 and P036D). If necessary, abnormal findings were confirmed by other MLPA probe kits, fluorescent in situ hybridization (FISH) or SNP array.

Results: A subtelomeric aberration was identified in 3.7% of patients (16/436). Details on subtelomeric aberrations and confirmation analyses are discussed.

Conclusions: This is the first study describing the presence of subtelomeric rearrangements in individuals with ID in Indonesia. Furthermore it shows that also in Indonesia such abnormalities are an important cause of ID and that in developing countries with limited diagnostic services such as Indonesia, it is important and feasible to uncover the genetic aetiology in a significant number of cases with ID.

Key-words: Intellectual disability (ID), subtelomeric rearrangements, multiplex ligation-dependent probe amplification (MLPA), Indonesia.

Introduction

Genetic causes of intellectual disability (ID) comprise (sub)microscopically chromosome abnormalities and monogenic diseases (Ropers, 2010). Microscopically visible numerical and structural abnormalities are the most common cause of ID. In a large meta-analysis review, a median rate of 9.5% was described (van Karnebeek et al., 2005). Apart from these microscopically visible chromosomal abnormalities, there are submicroscopic abnormalities that cannot be detected by conventional karyotyping. Abnormalities in the most distal ends of chromosomes, which harbour the highest gene concentrations in the human genome (Rudd, 2012), are difficult to identify on routine chromosome analysis, while they represent an important genetic cause of idiopathic ID. Therefore, testing for such rearrangements has turned out to be an important clinical evaluation step in the etiological diagnosis of unexplained ID cases (de Vries et al., 2003). In several studies, subtelomeric rearrangements were found to be associated with moderate to severe phenotypic abnormalities and turned out to be a significant cause of ID, with an estimated prevalence of 5-9% of cases in various populations (Wu et al., 2010; Christofolini et al., 2010; Jehee et al., 2011). To date, however, there is no data about the prevalence of subtelomeric rearrangements in Indonesia.

In a large number of Indonesian ID patients, the cause of ID could be established by conventional karyotyping or molecular testing for Fragile X syndrome (FXS), but the majority of cases still remained unexplained (Mundhofir et al., 2012a). Therefore, this study aimed at determining the prevalence of subtelomeric rearrangements and the clinical features in these ID individuals in Indonesia.

Methods

This research is an extension of previously reported studies on the identification of genetic causes of ID in Indonesia, where chromosomal aberrations and FXS were investigated in a large cohort of 527 Indonesian ID individuals from several special schools and institutions in Java Island, Indonesia. These previous studies revealed chromosomal abnormalities in 82 individuals and FXS in 9 individuals (Mundhofir et al., 2012a; Mundhofir et al., 2012b). In the present study, molecular testing of subtelomeric deletions and duplications was performed in the 436 as yet unresolved patients (278 males and 158 females). Informed consent was obtained from the parents or legal representatives and the study has been approved by the Ethical Board of our institute. All subjects underwent a standardized clinical examination including physical measurements and dysmorphological assessment.

DNA was isolated from peripheral blood using the salting out extraction procedure as described elsewhere (Miller et al., 1988). MLPA analysis was performed

as described previously (Schouten et al., 2002). Two probe-kits for subtelomeric chromosomal imbalances were used in these experiments: SALSA P070 and SALSA P036D MRC-Holland, Amsterdam, The Netherlands (<http://www.mrc-holland.com>). Each subtelomeric rearrangement was identified by at least one additional MLPA analysis using the SALSA P070 as the first level screening. Afterwards, SALSA P036D was utilized for confirmation of the aberration detected with the P070 kit. Rearrangements in specific regions were verified with SALSA kit P028, P023B, P340A or P096. The details of regions detected by each kit are available at <http://www.mlpa.com>. Amplification products were identified and quantified by capillary electrophoresis on an ABI 3730 genetic analyzer (Applied Biosystems, Foster City, CA, USA) using GeneMapper Software vs. 3.7 (Applied Biosystems). Statistical analyses were carried out using Microsoft Excel spreadsheets as described before (Koolen et al., 2004). Results were considered abnormal when the relative peak height ratio was below 0.70 or above 1.30.

When a deletion or duplication was detected in both MLPA kits, parents were tested for *de novo* occurrence. When parental DNA was not available, additional methodologies were performed for confirmation of the presence of the detected deletion or duplication. Fluorescence *in situ* hybridization (FISH) analysis was performed using commercially available probes (Vysis, Inc., Downers Grove, Illinois, USA) according to the manufacturer's recommendations. SNP array analyses were performed using the Affymetrix NsPl 250K SNP array platform (www.affymetrix.com). Copy number estimates were determined using the Copy Number Analyzer for Affymetrix Genechip Mapping (CNAG) software package version 2 (Nannya et al., 2005). The clinical data of all patients were reviewed and compared to other cases with a comparable aberration, described in the literature.

Results

The initial screening with the SALSA P070 probe kit showed a subtelomeric deletion and/or duplication in 23 of the 436 ID individuals (Table 1). In 20 of these samples the presence of the aberration could be confirmed by the SALSA P036D kit (cases 1-20, Table 1), while in the three remaining cases this was not possible and they were considered to be either artefacts or non-causative variants. Parental testing was possible in eight of the 20 cases (cases 1-8) and revealed a *de novo* occurrence of the subtelomeric imbalances in six cases (cases 1-6). These six aberrations were, therefore, considered to be pathogenic (Table 1 and 2). The phenotypes of cases 1 and 2 share many similarities with known cases with monosomy 18pter (Brenk et al., 2007) or a subtelomeric duplication of 9p24 (Ruiter et al., 2007) respectively. In cases 3, 4, and 5, a subtelomeric deletion appeared to be coexistent with a subtelomeric

duplication, which implicates the presence of a cryptic unbalanced translocation. The phenotype of case 3 was comparable to reported cases with either a 4p duplication (Brenk et al., 2007) or an 18p deletion (Cyr et al., 2011). Therefore, either dup 4pter or deletion 18pter (or both) could be contributing to the phenotype in the case 3. In case 4, the phenotype is most likely due to the deletion of 10pter, because of the phenotypic overlap with previously reported cases with a deletion of 10p15 (Lindstrand et al., 2010). The dysmorphic features of this case do not match the clinical description of another individual with a duplication of 9pter (Ruiter et al., 2007). In case 5, the 9pter/qter deletion/duplication is considered to be causative. The clinical details have been reported elsewhere (Mundhofir et al., 2012c). In case 6, a *de novo* deletion of the X/Yqter pseudo autosomal region 2 (PAR2) was detected, which was previously also described in phenotypically normal individuals (DGV; <http://www.tcag.ca/>). Therefore, we performed an additional SNP array analysis to enable a fairly precise determination of the size of the deletion and link it to the severity of the clinical features. To our surprise, this revealed a 2 Mb deletion on chromosome 22q11 that has previously been described in patients with a similar phenotype (Repetto et al., 2009). We therefore conclude that the phenotype of case 6 is not due to the *de novo* X/Yqter deletion but due to the 22q11 deletion.

In two cases (case 7 and 8) parental testing showed that the aberration was inherited from an unaffected parent. These aberrations were therefore considered to be familial variants that do not contribute to the phenotype in case 7 and 8. When parental samples were not available, we tried to confirm the presence of the detected subtelomeric aberration using additional methodologies (Table 1). The deletion of the entire Wolf-Hirschhorn critical region (WHSCR) in 4pter in case 9 was confirmed by MLPA with the SALSA P096 probe kit. Also the clinical features of this patient were consistent with Wolf-Hirschhorn syndrome. In case 10, two duplicated regions of chromosome 22 were identified, one in the 22q11.2 region (next to the centromere) and the other in the 22q13.3 region (telomere end of q arm of chromosome 22). The SALSA P023B probe kit was used to confirm these duplicated regions. Two duplications in one arm of the chromosome suggested a complex recombination. However, such recombination could not be identified in the routine analysis and warranted further characterization. Confirmatory analysis using SNP array showed that both duplications actually consisted of 2.6 Mb in 22q11.2 and 1.8 Mb in 22q13.33. Since microduplications of both 22q11.2 and 22q13.3 have been associated with highly variable phenotypic features (Feenstra et al., 2006; Rochebrochard et al., 2006), we suggest that in case 10 both duplications contribute to the phenotype.

Table 1. Subtelomeric rearrangements found in this study

Case	ID	Sex	MLPA result	Additional Method	Inheritance	Confirmation result	Conclusion
Case 1.	ID 398	F	del 18pter	-	<i>De novo</i>		Pathogenic
Case 2.	ID 190	M	dup 9pter	-	<i>De novo</i>		Pathogenic
Case 3.	ID 419	F	del 18pter/dup 4pter	SNP Array	<i>De novo</i>	Arr snp 4p16.3(SNP_A-2136377- >SNP_A-2289732)x3 Arr snp 18p11.32(SNP_A-1878089- >SNP_A-1781333)x1	Pathogenic
Case 4.	ID 386	F	del 10pter/dup 9pter	-	<i>De novo</i>		Pathogenic
Case 5.	ID 473	F	del 9pter/dup 9qter	SNP Array, FISH	Inherited	Arr snp 22q11.21(SNP_A-2108791- >SNP_A-2160861)x1	Pathogenic
Case 6.	ID 400	M	del X/Yqter	SNP Array	<i>De novo</i>	9p24.3 (SNP_A-2227623;SNP_A- 1898345)x1 9q34(SNP_A-4223541;SNP_A- 1874845)x3	Pathogenic ^{*)}
Case 7.	ID 460	F	del 16qter	-	Inherited		Non pathogenic
Case 8.	ID 207	M	dup 2pter	-	Inherited		Non pathogenic
Case 9.	ID 264	M	del 4pter	MLPA p096	NA	Deletion whole WHSCR	Pathogenic
Case 10.	ID 490	M	dup 22pter/qter	MLPA p023	NA	2.6 Mb duplication of 22q11 and a 1.8Mb duplication 22q13	Pathogenic
Case 11.	ID 502	M	del 9pter	SNP Array	NA	Arr SNP 9p24.3 (SNP_A-2227623- SNP_A-4235315)x1	Pathogenic
Case 12.	ID 366	M	del 9pter	-	NA		Pathogenic
Case 13.	ID 698	F	dup 15q11	MLPA me028	NA	Duplication between BP1-BP3	Pathogenic
Case 14.	ID 471	F	del 2qter	-	NA		Pathogenic
Case 15.	ID 462	F	dup 11pter/del Xpter	-	NA		Pathogenic
Case 16.	ID 641	M	del 12pter/dup 12qter	-	NA		Pathogenic
Case 17.	ID 640	M	del 8pter	-	NA		Possible pathogenic
Case 18.	ID 219	M	dup X/Ypter	-	NA		Possible pathogenic
Case 19.	ID 169	M	dup 16qter	-	NA		Possible pathogenic
Case 20.	ID 671	M	dup 16qter	-	NA		Possible variant
Case 21.	ID 237	M	dup 3q	-	NA		Possible variant
Case 22.	ID 269	M	dup 13q	-	NA		Variant
Case 23.	ID 363	F	del 9q	MLPA p340A1	NA	p340A1 not confirmed	Variant

*) SNP array revealed a 2 Mb deletion of chromosome 22q12. NA = Not available.

Table 2. Clinical features of individuals with subtelomeric rearrangements *).

Case no.	Case 1	Case 2	Case 3	Case 4	Case 6	Case 9	Case 10
MLPA Result	Del 18pter	Dup 9pter	Del 18pter/ dup 4pter	Del 10pter/ dup 9pter	Del X/Yqter	Del 4pter	Dup 22q
Gender	F	M	F	F	M	M	M
Age	16	24	11	10	12	24	25
Inheritance	De novo	De novo	De novo	De novo	De novo	NA	NA
ID Level	Mild	Mild	Moderate	Moderate	Moderate	Moderate	Mild
Hypotonia	Yes	No	Yes	Yes	Yes	Yes	No
Weight	32kg (<P3)	45 kg (<P3)	21 kg (<P3)	18 kg (<P3)	23 kg (<P3)	37 kg (<P3)	48 kg (P3-P25)
Height	145cm (<P3)	161 cm (<P3)	123 cm (<P3)	115 cm (<P3)	134 cm (P3)	143 cm (<P3)	161 cm (<P3)
OFC	50cm (<P3)	52.5 cm (<P3)	48 cm (<P3)	48 cm (<P3)	47 cm (<P3)	48 cm (<P3)	54 cm (P25)
Behaviour problems	introvert, shy	-	aggressive	ADHD	aggressive		
Other features							
Head / Face	prominent forehead, flat occiput, brachycephaly, flat face	sloping forehead, hypoplastic maxilla	brachycephaly,	brachycephaly, flat face, high forehead	long and narrow face	prominent glabella, greek helmet	high and broad forehead
Ocular region			ptosis, epicanthal folds, strabismus, downslanting palpebral fissure	hypertelorism, ptosis, hypoplastic orbita, strabismus, sparse eyebrows	strabismus, telecanthus,	hypertelorism	
Nose	flat nose, broad and deviated nasal bridge	globular nose	depressed nasal bridge	globular and broad base of nose	broad base of nose,	depressed nasal bridge, broad nose	
Ears	prominent anthelix	prominent and low set	posteriorly rotated, low set	prominent and posteriorly rotated		low set and simple, preauricular pits	prominent ears
Mouth region	unilateral cleft lip	micrognathia	downturned mouth, misaligned teeth	short philtrum, downturned mouth, pseudocleft	wide mouth, thick lips	bilateral cleft lip and palate, micrognathia	micrognathia
Thorax/ Abdomen			wide-spaced nipples	pectus excavatum		bulging of the thorax	
Extremities		single palmar crease, long fingers, flat feet, sandal gap	short 5 th finger, clinodactyly of 5 th finger	single palmar crease, clinodactyly of 5 th finger	single palmar crease	clinodactyly of 5 th finger	
Other Abnormalities			scoliosis, low posterior hairline, delayed speech	delayed speech		delayed speech	scoliosis

Table 2. (Continued)

Case no.	Case 11	Case 12	Case 13	Case 14	Case 15	Case 16	Case 17	Case 18
MLPA Result	Del 9pter	Del 9pter	Dup 15q11	Del 2qter	Dup 11pter/delXpter	Del12pter/dup12qter	del 8pter	dup X/Yp
Gender	M	M	F	F	F	M	M	M
Age	21	24	17	16	37	16	12	12
Inheritance	NA	NA	NA	NA	NA	NA	NA	NA
ID Level	Mild	Mild	Mild	Mild	Mild	Moderate	Mild	Mild
Hypotonia	No	No	No	No	No	Yes	No	No
Weight	52 kg (P25-P50)	54 kg (P25-P50)	40 kg (P3-P25)	55 kg (P75-P97)	34 kg (<P3)	44 kg (P3-P25)	30 kg (P25)	39 kg (P50)
Height	156 cm (<P3)	159 cm (<P3)	152 cm (P25)	146 cm (<P3)	127.5 cm (<P3)	153 cm (P3)	132 cm (P3)	144 cm (P3-P25)
OFC	54 cm (P25)	52 cm (<P3)	56 cm (P90-P97)	55 cm (P90-P97)	50 cm (<P3)	50 cm (<P3)	53.5 cm (P50)	55 cm (P90)
Behaviour problems			ADHD	Shy		Shy	ADHD	
Other features								
Head / Face	high forehead	face asymmetry, brachycephaly,	long face	asymmetry of the head		long and narrow face, brachycephaly, sloping forehead hypotelorism, almond shape palpebral fissure,	brachycephaly, high forehead	
Ocular region		arching eyebrows						
Nose			broad base of nose,				depressed nasal bridge	
Ears		small and low-set ears				low set ear	low set and posteriorly rotated ears	Prominent and large ears
Mouth region	Long philtrum, thin lips, micrognathia	thin upperlip, long philtrum,				small mouth, thick lips, high arched palate, micrognathia		
Thorax/ Abdomen Extremities		clinodactyly and sandal gaps	short 5 th finger and toe.	short hand, short metacarpal of digits 3-5, stubby hand, broad and short thumb, small and almost rudimentary of 3 rd and 4 th toe, hypoplastic nail	deviation of the long arm, clinodactyly and brachydactyly		sandal gaps	brachydactyly, clinodactyly, sandal gaps
Other Abnormalities	Cryptorchidism	low posterior hairline, kyphosis,			disproportionate short stature			

^a) clinical features of non-pathogenic cases (7,8), possible variant (19,20), variant (21,22,23) are not listed, as well as a case whose features are reported elsewhere (5).

In cases 11 and 12, a subtelomeric deletion of 9pter was identified. Case 11 showed a milder phenotype than previously reported cases (Hauge et al., 2008; Swinkels et al., 2008). In order to see if the deletion in case 11 was smaller than the deletions reported before, SNP array analysis was performed. The deletion appeared to be 11.8 Mb in size and it does not exceed the critical region of 9p syndrome (Hauge et al., 2008; Swinkels et al., 2008) this, therefore, explained the mild phenotype. In case 12, however, the patient showed some similarities to the reported cases (Hauge et al., 2008). Therefore, it is suggested that 9pter deletion in case 12 is causative and array analysis to determine the actual size of the deletion was unnecessary.

In case 13, a microduplication of 15q11 was identified. MLPA analysis of probes in the 15q11.2-15q15.1 region (MRC Holland kit P028) showed a duplication of the probes between Breakpoint 1 and Breakpoint 3 (including *TUBGCP5* and *APBA2*). The methylation specific analysis indicated that the interstitial duplication was of maternal origin. It is suggested, that the duplication explains mild ID and minor dysmorphic features in case 13, since duplications of 15q11 are associated with a highly variable phenotype (Bolton et al., 2001).

In cases 14-20, parental testing and additional testing were not performed. This was due to the fact that in some cases, materials for additional testing were unavailable. Another reason was that the clinical features of the patient showed some similarities to the reported cases; therefore, additional testing was considered unnecessary. In case 14, a subtelomeric deletion of 2qter was identified. This patient showed shortening of the metacarpal bones which occurs in the majority of 2q37 patients (Felder et al., 2009). It is suggested, therefore, that the deletion in this case is causative. In case 15, a subtelomeric deletion of Xpter and a subtelomeric duplication of 11pter were identified. The presence of both a deletion and a duplication suggests an unbalanced translocation. The Xpter probes in the P070 and P036D MLPA kits are both located within the *SHOX* gene. Deletions of this gene are associated with Leri-Weill Dyschondrosteosis (LWD) and idiopathic short stature (ISS) (Barroso et al., 2010; Hirschfeldova et al., 2012). A duplication of 11pter has been reported in a case of Silver-Russell syndrome (Eggermann et al., 2010). Therefore, both the dup 11pter and the deletion Xpter could be contributing to the short stature of this individual's phenotype.

In case 16, the subtelomeric deletion of 12pter accompanied by a subtelomeric duplication of 12qter suggests that the recombinant chromosome resulted from a pericentric inversion in one of the parents. Unfortunately, his parents were unavailable to test. Lagier-Tourenne et al. (2004) reported two cases of microscopically visible recombinant chromosome 12 and reviewed all previously reported cases as well as cases with pure cytogenetic deletion of 12pter and duplication of 12qter. Compared to

these previously reported cases with larger abnormalities, our patient showed a milder phenotype such as minor facial dysmorphisms and mild ID (Table 2). It is suggested that the terminal duplication/deletion of chromosome 12 in this patient was smaller than previously reported and could contribute to the phenotype.

In case 17, a subtelomeric deletion of 8pter was identified. He showed mild ID with minor facial dysmorphisms. A subtelomeric 8pter deletion is rare, and only few cases have been reported (de Vries et al., 2003). Wu et al., (2010) reported a patient with a very small deletion in terminal 8pter with ID, microcephaly and minor facial dysmorphisms. We therefore conclude that the clinical features of case 17 are most likely due to the deletion of 8pter.

In case 18, a duplication of the probes in the *SHOX* gene in the pseudo-autosomal region 1 (PAR1) Xpter/Ypter was identified. *SHOX* duplications limited to PAR1 appear to be rare, and the associated phenotype is highly variable (Thomas et al., 2009; Hirschfeldova et al., 2011). *SHOX* gene defects, either a deletion or duplication, were associated with LWD and ISS. It has to be noted, however, that the effect of a duplication is ambiguous (Hirschfeldova et al., 2012). Consequently, the clinical features associated with such duplication were likely to be under-ascertained (Hirschfeldova et al., 2011). We are uncertain, therefore, whether the duplication of this gene contributed to the clinical phenotype or not.

In cases 19 and 20, a subtelomeric duplication of 16qter was identified. A 16qter submicroscopic microduplication is rarely reported. Ravnan et al., (2006) reported five cases with a duplication 16qter in which the duplicated signal was adjacent to the 18p subtelomere probe signal. In two cases the recombination appeared to be inherited from unaffected parents, and these were considered to be variants. Therefore, in the other three cases the recombination was also regarded to be a variant although parental samples were not available (Ravnan et al., 2006). It cannot be ruled out, however, that the duplication in cases 19 and 20 is contributing to their phenotype, since 16qter is a gene-rich region. More than ten genes are present in the ~500 kb proximal to 16qter. Some of these (*NULP1*, *TUBB3*, and *AFG3L1*) are expressed in the brain (Zou et al., 2007), and it is possible that overexpression of these genes contributes to ID.

Discussion

This is the first study identifying subtelomeric chromosomal aberrations in Indonesian ID individuals. Overall, subtelomeric copy number rearrangements were established in 20 samples, explaining the phenotype of 16 cases. Therefore, a detection rate of 3.7 % (16/436) was obtained, of which 31% (5/16) was found to have a complex rearrangement/unbalanced translocation, 44% (7/16) had a simple deletion

and 25% (4/16) had a simple duplication. In addition, the subtelomeric rearrangements contributed as genetic cause of ID in 3% (16/527) of cases in the whole cohort. The deletions, including the complex rearrangements, involved nine different subtelomeric regions (2q, 4p, 8p, 9p, 10p, 12p, 18p, X/Yp, X/Yq); and duplications, including complex rearrangements, involved eight subtelomeric regions (4p, 9p, 9q, 11p, 12q, 15q11, 22p, X/Yp).

The detection rate of chromosomal subtelomeric rearrangements in this study is 3.7 % (16/436) which is well within the range of 2.5% previously reported by Ravnan et al., (2006) and 4.4% as reported by van Karnebeek et al., (2005). Five individuals (5/16; 31%) are suggested to have an unbalanced translocation that was not detected by routine cytogenetic analysis. In two of these the translocation was shown to be *de novo* (case 3 and 4); in two individuals, parental samples were unavailable (case 15 and 16); and inherited translocation was demonstrated in one case (case 5). The prevalence of these cryptic imbalances in our ID population is in the range of a previous study conducted by Wu et. al., (2010), who reported such rearrangements in 21.7% (5/23) and the study of Jehee et al., (2011) which reported such rearrangements in 42.1% (8/19) (Wu et al., 2010; Jehee et al., 2011). The considerably high rate of unbalanced translocations observed in this study might be explained by the fact that we did not use high-resolution banding, which could have detected most 'cryptic' subtelomeric anomalies (Mundhofir et al., 2012a).

Although the MLPA method is capable of revealing subtelomeric rearrangements, the clinical significance of each rearrangement should be interpreted carefully, particularly for cases in which the clinical features are different from previously reported cases. In case 6, for example, the *de novo* subtelomeric X/Yqter deletion could not explain the clinical features when compared to the previously reported cases (Parvari et al., 1999; Ravnan et al., 2006). Furthermore, in the DGV it is reported that rearrangements in this region can be detected in phenotypically normal individuals as well. Subsequent SNP array in this patient identified another abnormality which explained his clinical phenotype.

To conclude, this is the first large-scale study of the detection of submicroscopic subtelomeric aberrations in Indonesian patients with ID. This study shows that subtelomeric rearrangements are an important cause of ID in Indonesia and its prevalence does not differ from previously reported studies in the Western world. Since diagnostic facilities for this kind of abnormalities are not yet available in Indonesia, the implementation of this technique in a routine diagnostic setting will help to establish a genetic diagnosis in individuals with ID, and will improve the possibilities for genetic counselling to the families involved. To establish an adequate diagnosis is of crucial importance for the patients and their families. Therefore, diagnostic facilities for

genetic diseases need to get a higher priority in Indonesia, similar to those for common infectious diseases and nutritional problems.

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4.2

Monosomy 9pter and trisomy 9q34.11qter in two sisters due to a maternal pericentric inversion

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Chapter 5.

The aetiology in a subgroup of ID individuals suspected of having a specific syndrome

- 5.1 Mowat–Wilson syndrome: The first clinical and molecular report of an Indonesian patient (Case Rep Genet. Article ID 949507,doi:10.1155/2012/949507)
- 5.2 Molecular analyses on Indonesian individuals with intellectual disability and microcephaly (submitted)

Mowat–Wilson syndrome: The first clinical and molecular report of an Indonesian patient

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Abstract:

Mowat–Wilson syndrome (OMIM 235730) is a genetic condition characterized by moderate-to-severe intellectual disability, a recognizable facial phenotype and multiple congenital anomalies. The striking facial phenotype in addition to other features such as severely impaired speech, hypotonia, microcephaly, short stature, seizures, corpus callosum agenesis, congenital heart defects, hypospadias and Hirschprung disease are particularly important clues for the initial clinical diagnosis. All molecularly confirmed cases with typical MWS have a heterozygous loss-of-function mutation in the zinc finger E-box protein 2 (*ZEB2*) gene, also called *SIP1* (Smad-interacting protein 1) and *ZFHX1B*, suggesting that haploinsufficiency is the main pathological mechanism. Approximately 80% of mutations are nonsense and frameshift mutations (small insertions or deletions). About half of these mutations are located in exon eight. Here, we report the first Indonesian patient with Mowat-Wilson syndrome confirmed by molecular analysis.

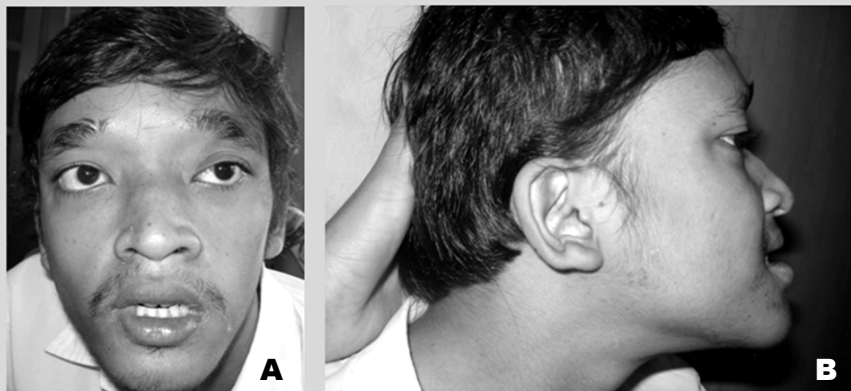
Introduction

Mowat–Wilson syndrome (MWS; OMIM 235730) is a rare genetic condition described by Mowat et al. in 1998, who reported a series of six children with intellectual disability (ID), striking facial features, and variable multiple congenital anomalies (MCA) (Mowat et al., 1998). All molecularly confirmed cases with typical MWS have a heterozygous loss of function mutation in the zinc finger E-box protein 2 (*ZEB2*) gene, also called *SIP1* (Smad-interacting protein 1), and *ZFHX1B* (Wakamatsu et al., 2001). To date, about 200 molecularly proven MWS cases with over 100 different *ZEB2* mutations have been reported (Evans et al., 2012).

The facial features are the most important diagnostic clue for the initial clinical diagnosis and provide a hallmark for *ZEB2* mutation analysis (Garavelli and Mainardi, 2007). Establishing a molecular diagnosis is important for the patients and their families as it allows reliable genetic counseling for their families and a better clinical management of the patients. Here, we report the first Indonesian patient with molecularly confirmed MWS.

Case presentation

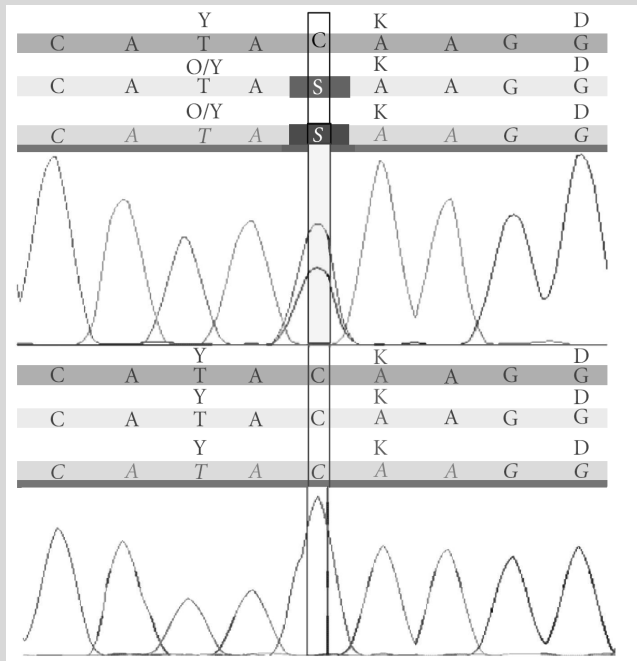
The patient was a nineteen-year-old male with severe ID. He was the third son of nonconsanguineous, healthy, Javanese parents and family history was unremarkable. The patient was born at term after an uneventful pregnancy with a weight of 3200 g (25th centile) and length 50 cm (50th centile). He showed hypotonia and delayed developmental milestones. He started to sit at 20 months of age. At two years of age, he developed recurrent generalized seizures and was commenced on valproic acid, which brought his epilepsy under control. He started to walk at four years of age and spoke his first words at the age of five years. He had recurrent otitis media. Speech consisted of only a few words and he often communicated using sign language. He showed happy behavior with frequent smiling. In addition, he showed repetitive hand movements. On physical examination, his weight was 45 kg (<3rd centile), height 161 cm (<3rd centile) and head circumference 53 cm (<3rd centile). Facial dysmorphisms included a long face, deep-set eyes, large eyebrows with medial flaring, hypertelorism, strabismus, saddle nose with prominent rounded nasal tip, prominent columella, low-set and posteriorly rotated ears, uplifted ear lobules, a prominent narrow pointed chin, a small mouth and prognathism (Figure 1). In addition, he had tapered and slender fingers, prominent interphalangeal joints, and bilateral pes planus. Generalized hypotonia and hyperreflexia were observed. Heart auscultation was normal.

Figure 1. Photograph of Indonesian patient with Mowat-Wilson syndrome.

Our patient in his 19 years of age showed striking facial gestalts of MWS such as large eyebrows with medial flaring (a) and uplifted ear lobules (b). Other dysmorphisms such as long face, deep-set eyes, upward slanting palpebral fissures, hypertelorism, strabismus, saddle nose with prominent rounded nasal tip, prominent columella, low-set and posteriorly rotated ears, prominent and triangular pointed chin, small mouth, full lips, and prognathia are noted.

The individual was part of a larger series of 527 Indonesian individuals with ID from schools and institutions, whose conventional karyotyping, *FMR1* gene analysis and subtelomeric MLPA were normal (Mundhofir et al., 2012). Based on the clinical features, MWS was suspected. Therefore, molecular analysis of the *ZEB2* gene was warranted. Sanger sequencing of all coding exons and surrounding splice sites of the *ZEB2* gene was performed as described below. The genomic DNA reference sequence was NM_014795.2. PCR of exon eight was performed using primers CTTTACTTGGGTTTCCCACC (forward) and GGGGCTTGTCATTCCTT (reverse). One hundred nanograms of DNA solution (1 μ L) was added into PCR mixture, which contained 7.6 μ L of 360 PCR master mix (Applied Biosystem), 0.5 μ L of primers working solution, and 6 μ L of H₂O. Amplification was performed using PCR System 9700 (Applied Biosystem) with the following protocol. PCR was initiated by 10' denaturation at 95°C, followed by 35 PCR Cycles (30" 95°C, 30" 60°C, 60" 72°C) and 7' final elongation at 72°C. The result was analyzed on ABI 3730 analyzer (Applied Biosystem). Sequence result was compared to published reference sequence (rs148709333) using SEQPilot software version 3.2.1.0 (JSI medical system). In exon eight, a nonsense mutation has been detected, changing a TAC codon (coding for a tyrosine) into a TAG stopcodon; c.1965C>G (p.(Tyr652*)) (nomenclature according to the HGVS guidelines; <http://www.hgvs.org/mutnomen/>) (Figure 2). To our knowledge, this mutation has not been reported before.

Figure 2: Electropherogram of molecular analysis in the patient sample.



The upper panel shows the heterozygous c.1965C>G (p.Tyr652X) mutation and the lower panel shows the wild type (control). The "S" on the electropherogram represents the C/G heterozygote.

Discussion

This is the first report of an Indonesian individual with MWS confirmed by molecular genetic testing. Although nonsense mutations account for more than 40% of known *ZEB2* mutations and approximately 50% of these are localized in exon eight (Dastot-Le Moal et al., 2007), the particular mutation detected in the patient described in this paper (c.1965C>G; p.Tyr652*) has not been reported before.

Most clinical features of our patient, who had severe ID, a distinct facial gestalt, microcephaly, and seizures, are consistent with those described in the literature (Table 1). Brain imaging and echocardiography could not be performed since he is living in the country with minimal health facilities. Symptoms of Hirschprung disease (HSCR) such as constipation, dysphagia, and poor appetite were not reported in our patient, but the prevalence of these symptoms in other publications ranged from the majority of individuals (Mowat et al., 1998; Wakamatsu et al., 2001) to 50% of cases (Dastot-Le Moal et al., 2007; Garavelli and Mainardi, 2007). Early diagnosis, intervention, and targeted management are necessary for a better health and life quality of individuals with MWS. However, as this syndrome is rare and recently described, the knowledge of

the clinical complications and natural history is still developing (Mowat and Wilson, 2010).

Table 1: Clinical features of our patient compared to those in published cases of MWS with proven *ZEB2* mutations.

Clinical features	Our patient	Mowat-Wilson syndrome*
<i>ZEB2</i> mutations	+	100%
Intellectual disability	+	100%
Typical facial gestalt	+	97%
Microcephaly	+	81%
Seizures	+	73%
HSCR	-**	57%
CHD	-**	52%
Hypospadias	-	52%
Short stature	+	46%
Hypoplasia or agenesis of CCA	NT	43%
Cryptorchidism	-	36%
Constipation	-	26%
Pyloric stenosis	-	4.7%
Eye anomalies	-	4.1%
Cleft palate	-	2.9%

* Adapted from Garavelli and Mainardi (2007).

**Symptoms not observed although the gold standard diagnosis has not been performed.

NT: not tested, HSCR: Hirschprung disease, CHD: congenital heart defect, CCA: Corpus Callosum.

In summary, we report the first Indonesian MWS case with a novel *ZEB2* mutation. Our patient showed similar dysmorphism to previously reported cases, although several major associated features were not present such as HSCR, congenital heart defect (CHD) and hypospadias. Despite the availability of molecular diagnostic tests in several parts of the world, the recognition of clinically well defined syndromes will remain very important in countries with limited diagnostic facilities such as Indonesia. The publication of cases with recognizable facial features is therefore of great importance in order to make local pediatricians aware of rare conditions like Mowat-Wilson syndrome, allowing more clinical diagnoses in the future.

Conflict of interest

The authors have no conflict of interest to declare

Acknowledgements

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5.2

Molecular analyses in Indonesian individuals with intellectual disability and microcephaly

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Submitted

Abstract

Background

Intellectual disability (ID) often coincides with an abnormal head circumference (HC). Since the HC is a reflection of brain size, abnormalities in HC may be a sign of a brain anomaly. Although microcephaly is often secondary to ID, hereditary (autosomal recessive) forms of primary microcephaly (MCPH) also exist that result in ID.

Objective

To investigate mutations in MCPH genes in patients with ID and microcephaly.

Methods

From a population of 527 Indonesian ID individuals, 48 patients with microcephaly (9.1%) were selected. These patients were previously found to be negative upon conventional karyotyping, *FMR1* gene analysis and subtelomeric deletion and duplication MLPA. Sanger sequencing for *ASPM* and *WDR62* was performed in all 48 samples, while sequencing for *MCPH1*, *CDKRAP2*, *CENPJ* and *STIL* was conducted only in 20 samples with an OFC below -4SD.

Results

In all genes investigated, 66 single nucleotide polymorphisms (SNPs) and 15 unclassified variants which were predicted as unlikely to be pathogenic (UV2) have been identified. Possible pathogenic variants (UV3) have only been identified in *ASPM* and *WDR62*. However, since none of the patients harboured compound heterozygous likely pathogenic mutations, no molecular diagnosis of MCPH could be established. Interestingly, one of the patients harboured the same variants as her unaffected monozygotic twin sister, indicating that our cohort includes a discordant twin.

Conclusions

This is the first study to identify genetic causes of MCPH in the Indonesian population. The absence of causative pathogenic mutations in the tested MCPH genes might originate from several factors. The identification of UV2 and UV3 variants as well as the absence of causative pathogenic mutations calls for further investigations.

Keywords: Intellectual disability (ID), microcephaly, MCPH genes, Sanger sequencing, Indonesia

Introduction

Intellectual disability (ID) has been estimated to have a prevalence close to 3% worldwide and has a variety of genetic causes (van Bokhoven, 2011). In patients with ID, an abnormal head circumference (HC) is often another main sign (Mochida, 2009; Kaindl et al., 2010). Since the HC or occipitofrontal circumference (OFC) is a reflection of brain size, abnormal head circumference may be a sign of a brain anomaly. Microcephaly is commonly classified as HC below minus two standard deviations (SD) or below the 2nd centile for the patient's age and gender (Opitz and Holt, 1990; Leviton et al., 2002). Its incidence at birth is between 1.3 and 150 per 100,000 live births (Kaindl et al., 2010).

The aetiologies of microcephaly can be divided into genetic causes and environmental insults to the brain during prenatal, perinatal, or early postnatal period (Tarrant et al., 2009). Primary microcephaly/congenital microcephaly is described as a static developmental abnormality which presents at birth or as early as 32 weeks of gestation (Woods, 2004). Secondary microcephaly is considered as a progressive neurodegenerative condition where the head circumference at birth is within normal range and microcephaly develops thereafter (Woods, 2004; Abuelo, 2007). The presence of microcephaly at birth is one of the signs of genetic microcephaly (Mochida, 2009). Until recently, eight loci associated with autosomal recessive primary hereditary microcephaly (MCPH1-MCPH8) have been found and causative mutations have been identified in the 8 MCPH genes. Details of known autosomal recessive primary microcephaly (MCPH) genes are summarized in Table 1.

Several mutations have been identified in these loci in different countries (Wollnik, 2010; Soltani Banavandi et al., 2012; Hussain et al., 2012b). However, as no genetic analysis has been performed in the Indonesian population, this study aimed at investigating the presence of mutations in those genes. The understanding of possible genetic causes of microcephaly associated with ID in the Indonesian population is expected to assist appropriate aetiological diagnosis and genetic counselling in the affected individuals and their families.

Table 1. Known autosomal recessive primary microcephaly (MCPH) loci, the chromosome location, the causative gene, incidence, and proposed function

Locus	Chromosome	Gene	Linked families ^{a)}	Mutations Identified ^{a)}	Frequency ^{a)}	Function	Phenotype ^{b)}	References
MCPH1	8p23	<i>MCPH1</i> or <i>Microcephalin</i>	3	3	1.1%	DNA damage repair, chromosome condensation	PM, PCC	(O'Driscoll et al., 2006; Trimborn et al., 2010)
MCPH2	19q13.12-q13.2	<i>WDR62</i>	9	6	2.3%	Positioning of spindle poles, neural precursor for cerebral cortex growth	PM, PCT, HCC, PG	(Yu et al., 2010; Nicholas et al., 2010; Bilguvar et al., 2010)
MCPH3	9q33.2	<i>CDK5RAP2</i>	3	2	0.8%	Regulation of microtubule, centrosome maturation	PM, SC	(Bond et al., 2005; Fong et al., 2008)
MCPH4	15q15-q21	<i>CEP152</i>	5	1	0.4%	Centriole duplication	PM	(Guernsey et al., 2010; Kalay et al., 2011)
MCPH5	1q31.3	<i>ASPM</i>	101	96	36.6%	Spindle pole organization and orientation	PM	(Kouprina et al., 2005; Zhong et al., 2005)
MCPH6	13q12.12	<i>CENPJ</i>	8	6	2.3%	Centriole length control, microtubule dynamics	PM, SC	(Hung et al., 2004; Bond et al., 2005)
MCPH7	1p33	<i>STIL</i>	5	3	1.1%	Spindle organization, cell cycle progression	PM	(Kumar et al., 2009; Vulprecht et al., 2012)
MCPH8	4q12	<i>CEP135</i>	1	2	0.8%	Scaffold for centriole, microtubule assembly, procentriole formation	PM	(Hussain et al., 2012a)
Unlinked ^{c)}			143		54.6%			(Mahmood et al., 2011; Hussain et al., 2012b)

a) Data have been calculated from published families (Kumar et al., 2009; Mahmood et al., 2011; Hussain et al., 2012a; Hussain et al., 2012b)

b) PM=primary microcephaly; PCC=premature chromosome condensation syndrome; PCT=pachygyria with cortical thickening; HCC=hypoplasia of the corpus callosum; PG=polymicrogyria; SC=Seckel syndrome.

c) Unlinked=not linked to any of eight MCPH loci (Hussain et al., 2012b; Mahmood et al., 2011)

Methods

Samples were selected from a cohort of 527 ID individuals in whom physical examinations, dysmorphology assessments, blood collections and several genetic screenings were performed (Mundhofir et al., 2012). Peripheral duplicate blood samples in EDTA and Heparin were collected from these individuals. Several genetic screenings including cytogenetic analysis, *FMR1* gene and subtelomeric rearrangements were carried out (Mundhofir et al., 2012). A total of 48 individuals whose cytogenetic analysis, and tests for *FMR1* gene and subtelomeric rearrangements turned out to be normal and whose HC measurement was less than -2 standard deviation (microcephaly) measured with the Nellhaus charts (Nellhaus, 1968) were included in this study. Informed consent was obtained from their parents or legal representatives; and the study was approved by the Ethical Board of the University of Diponegoro/Kariadi Hospital Semarang, Indonesia. Clinical characteristics of the studied population are summarized in Table 2.

Table 2. Characteristics of ID individuals with microcephaly ($n = 48$)

Characteristics	<i>n</i>	%
Sex		
Female	23	47.9
Male	25	52.1
Level of ID		
Mild	22	45.8
Moderate	17	35.4
Severe	9	18.8
OFC		
-2SD< <i>n</i> <-3SD	6	12.5
-3SD< <i>n</i> <-4SD	22	45.8
<-4SD	20	41.7
Associated abnormalities		
Epilepsy	7	14.6

Genomic DNA of each patient was isolated using salting out method DNA extraction as described elsewhere (Miller et al., 1988) with slight modification. Sanger sequencing for *ASPM* and *WDR62* was performed in all 48 ID samples. Considering the low prevalence of the remaining MCPH genes (*MCPH1*, *CDKRAP2*, *CENPJ* and *STIL*) in the beginning of our study the sequencing of these 4 genes was conducted only on 20 samples with the lowest OFC (below -4SD). *CEP152* and *CEP135* were not sequenced in this study as they have recently been discovered. PCR products of all coding exons were generated using standard techniques. Sequences of the PCR primers are available upon request. In brief, 1 μ L DNA (~100 ng) from each sample was

diluted in a PCR mix solution, consisting of 7.6 μ L of 360 PCR mix (Life Technologies, Foster City, USA), 0.5 μ L of each primer (Biolegio, Nijmegen, The Netherlands) and 6 μ L of purified water. The mixture was amplified in a 9700 Veriti PCR system (Life Technologies, Foster City, USA) using the following protocol: initial denaturation at 95°C for 10' was followed by 35 cycles of 94°C for 30", 60°C for 60" and 72°C for 60", and ended with elongation at 72°C for 1'. Subsequently, the PCR products were purified using Millipore columns according the protocol recommended by the manufacturer (Merck Millipore, New Jersey, USA). One μ L of purified PCR product was mixed with M13 primer (forward or reverse). Amplification and purification step were performed according the protocol recommended by the manufacturer and was then analyzed on an ABI 3730 XL sequencer (Life Technologies, Foster City, USA).

Sequence variations in the DNA of the patients were compared to the reference sequence in *Ensembl* assembly GRCh37 (GCA_000001405.6) from the Genome Reference Consortium (<http://www.ncbi.nlm.nih.gov/projects/genome/assembly/grc/human/index.shtml>) using the following reference sequences: *ASPM* (NM_018136.4) *WDR62* (NM_001083961.1), *MCPH1* (NM_024596.3), *CDK5RAP2* (NM_018249.4), *CENPJ* (NM_018451), and *STIL* (NM_001048166). The DNA sequencing data were analyzed with SEQPilot software version 3.2.1.0 (Softgenetics, Pennsylvania, USA). Variants were further analyzed using Alamut 2.0 mutation interpretation software (Interactive Biosoftware, Rouen, France). The impact of protein alteration was calculated using Align-GVGD method (<http://agvgd.iarc.fr/>), SIFT (<http://sift.jcvi.org/>), and Polyphen (<http://genetics.bwh.harvard.edu/pph2/>). The variants found in this study were then classified according to the guidelines of the UK Molecular Genetics Society (CMGS) and the Dutch Society of Clinical Genetic Laboratory Specialists (*Vereniging Klinisch Genetische Laboratoriumspecialisten*). The classifications are: Class 1 (UV1)=Certainly non pathogenic; Class 2 (UV2)=Unlikely to be pathogenic; Class 3 (UV3)=Likely to be pathogenic and Class 4 (UV4)=Certainly pathogenic.

For one of the patients and her family, an identifier test was conducted in a follow up study using AmpFISTR Identifier Direct PCR Amplification Kit (Life Technologies, Foster City, USA) using the manufacturer's protocol.

Results

In this study, a total of 186 amplicons were sequenced, including the sequencing of *ASPM* and *WDR62* in 48 samples and *MCPH1*, *CDKRAP2*, *CENPJ* and *STIL* in 20 samples. In *ASPM* and *WDR62* a total of 26 unclassified variants have been identified, 15 of which were predicted as unlikely to be pathogenic (UV2), 10 were

predicted as likely to be pathogenic (UV3) and one which could not be classified. In addition, 15 and 13 single nucleotide polymorphisms (SNPs) (classified as certainly not pathogenic (UV1)) were identified in the *ASPM* and *WDR62* gene, respectively. In the remaining genes, only known SNPs have been identified: 15 *MCPH1* variants, 14 *CDKRAP5* variants, 6 *CENPJ* variants and 3 *STIL* variants. Since MCPH is an autosomal recessive disease, only the presence of homozygous or two compound heterozygous mutations would explain the phenotype. Only one of the patients harboured two *ASPM* variants that were classified as UV3. A follow up study in the family was needed to investigate if these variants reside on different chromosomes. Sequence analysis of the variants in both parents and a twin sister revealed that both variants were present in the unaffected father and twin sister, indicating that they are present on the same chromosome and do not explain the phenotype in the patient. To exclude the possibility of an unidentified mutation in the second (maternal) allele, an identifiler test was performed to examine whether the twins were dizygotic or monozygotic. Interestingly, the result showed that the twin sisters were monozygotic, which makes autosomal recessive inheritance of the condition in this family highly unlikely.

Discussion

This is the first study to identify the genetic cause of microcephaly associated ID in the Indonesian population. Albeit pathogenic mutations were not found, the identification of numerous single nucleotide polymorphisms (SNPs) may be useful in future association studies in our population. In addition, it has not been excluded that some of the UV3 variants might turn out to be pathogenic.

An interesting finding in this study included the identification of a monozygotic twin in which only one of the sisters was affected. Underlying mechanisms to contribute to this so called twin discordance phenomenon are alterations in gene structure and expression during meiosis and/or mitosis, epigenetic changes or phenocopy (Miller et al., 2012). As the variants were also identified in the unaffected father and the unaffected monozygotic twin sister, autosomal recessive primary microcephaly turned out to be a highly unlikely diagnosis in the affected sister. Therefore, it is expected that the disease is more likely caused by a *de novo* mutation with a low recurrence risk for future children of the parents. This knowledge is not only useful for counselling in the family, but paves the way for future identification of the genetic defect in this family, e.g. by exome sequencing comparing the genotypes of the two twin sisters.

The absence of causative pathogenic mutations in the MCPH genes investigated in this study might be due to several reasons. First, the microcephaly and

ID in these individuals might not be genetic. Several non-genetic factors, such as undernutrition, infections, ischaemia, metabolic disturbances, exposure to toxic substances, and trauma are known to play a role in microcephaly (Tarrant et al., 2009; Jauhari et al., 2011). As there is limited information on the onset of the microcephaly in these individuals (birth HC measurement is hardly available), non-genetic factors could not be ruled out. Second, microcephaly in these individuals may due to secondary microcephaly. Third, there is a possibility that causative mutations are present in the tested genes, but were missed or were not recognized by the methods utilized in this study. For example, the presence of exonic or whole gene deletions has not been investigated, since only direct sequencing has been performed (Thornton and Woods, 2009; Mahmood et al., 2011). Fourth, there is a chance that the microcephaly may result from mutations in genes that have not yet been discovered, since more than half of reported families with MCPH have not been linked to any of the eight MCPH loci (see Table 1). Fifth, there is a possibility that the genetic defects occur in *CEP152* and *CEP135* that were not investigated in this study.

The overall frequency of microcephaly with ID in this study was 9.1% (48/527). This is comparable to studies performed in other Asian ID populations such as the Indian (9.8%) (Jauhari et al., 2011) and Israeli (15.4%) (Waternberg et al., 2002) populations. However, the frequency of microcephaly in this study might have been overrated. The mean value of height and weight of Indonesian children in the nationwide survey was smaller compared to the United States of America Centers for Disease Control and Prevention growth charts (Batubara et al., 2006). Considering the correlation between height and weight and head circumference, Indonesian individuals are expected to have a smaller HC. In addition, in a pilot study conducted in Semarang, Central Java, the mean value of the head circumference of Indonesian normal school children aged 7-12 was smaller than the normal range of the Nellhaus charts (unpublished observations). The mean values of boys ($n=128$) were between the 3rd and 50th centile while of the girls ($n=146$) these were between the 10th and 50th centile. For a better estimation of the incidence of microcephaly, future studies are required to establish a normal HC chart for the Indonesian population.

To conclude, this is the first large-scale study to identify genetic causes of microcephaly with ID in the Indonesian population. This study shows that the frequency of microcephaly and ID is not much different from previously reported studies in Asian populations. The absence of causative pathogenic mutations in the tested MCPH genes might originate from several factors such as non-genetic factors, secondary microcephaly and mutations in other genes that were not investigated in our study. The identification of UV2 and UV3 variants as well as the absence of causative pathogenic mutations calls for further investigations, such as sequencing of other MCPH genes

(*CEP152* and *CEP135*) and whole exome sequencing study, preferably in a larger and better defined sample.

Acknowledgments

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Note

The data of the SNPs and other variants are available upon request.

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6

Chapter 6. General discussion and the establishment of a diagnostic protocol for the Indonesian setting

6.1. Diagnostic investigations in Indonesian ID individuals

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6.4.1. Recommendations

6.4.2. Future directions

The work described in this thesis is a continuation of the first screening study of ID in a large cohort of Indonesian individuals (Hussein, 1998; Faradz et al., 1999). The previous study was carried out using conventional cytogenetic and *FMR1* gene analyses with the main focus on males. This thesis, however, characterizes and discusses the results of a comprehensive genetic-aetiological survey of individuals with ID carried out in the nation and provides a protocol for diagnostic work up in Indonesia.

6.1. Diagnostic investigation in Indonesian ID individuals

6.1.1. The yields of aetiological genetic assessments in the Indonesian ID population

The identification of several genetic abnormalities has revolutionized the diagnostic process of patients with ID. Nowadays, the development and implementation of novel methodologies have shown that genetic abnormalities are important causes of ID. As genetic aetiological tests are rarely performed, we carried out some genetic analyses in undiagnosed Indonesian ID individuals. We performed cytogenetic analysis using conventional karyotyping with confirmation by some stand-alone molecular tests. We identified cytogenetic abnormalities in 16.5% (87/527) patients, demonstrating that cytogenetic abnormalities are one of the major genetic causes of ID in our cohort (**Chapter 2**).

In order to establish the frequency of FXS in male and female ID Individuals, we investigated the promoter region of the *FMR1* gene in Indonesian ID individuals using PCR based fragment length analysis, with confirmation of expanded alleles by Southern blot and repeat-primed PCR methods. The detection rate of FXS was 1.7% (9/527) with a frequency in males and females of 1.5% (5/329) and 2% (4/198), respectively. Moreover, we documented relatives with the same disorder, demonstrating that FXS is an important cause of inherited ID in our cohort (**Chapter 3**).

Deletions and duplications of subtelomeric regions are an important cause of unexplained cases of ID. These abnormalities have not been investigated in Indonesian ID individuals. In order to determine the frequency of subtelomeric rearrangements in a cohort of Indonesian ID individuals, who had a normal karyotype and who were FXS negative, we performed MLPA analysis with probes in all subtelomeres. We identified clinically relevant rearrangements in 3% (16/527) of them, demonstrating that subtelomeric rearrangements are important causes of unexplained ID in our cohort (**Chapter 4.1**). Moreover, in one of these individuals, we identified familial monosomy 9pter and trisomy 9q34.11qter in two sisters, due to a maternal pericentric inversion (**Chapter 4.2**).

The latest meta-analysis study of genetic screening yields in the ID individuals comprised 367 out of 7,000 genetic screening studies between 1980–2009. It was reported that the frequency of chromosome aberrations was 4.2% and 18.6% for non-syndromic and syndromic ID, respectively.

In their study, the detection rate of FXS was 2%, and the frequency of subtelomeric rearrangements was 0.5% and 7.4% for individuals with milder and

moderate/severe impairments, respectively (Michelson et al., 2011). The detection rates of chromosome aberrations, FXS, as well as subtelomeric deletions, in our study, are comparable with this meta-analysis.

A total of 72 out of 74 (97.3%) of DS individuals were diagnosed clinically, whereas 7 of 9 individuals (77.8%) were clinically suspected of having FXS. Although Down syndrome and FXS can be diagnosed on clinical grounds, these affected ID individuals in this cohort had not been cytogenetically and molecularly investigated. This explains why a relatively high frequency of Down syndrome and FXS was identified in this study (14% and 2% respectively) (**Chapter 2 and 3**).

In some of the ID individuals in our cohort, the combination of clinical features suggested the presence of a specific syndrome. In order to confirm this hypothesis, we performed molecular testing of the genetic defect involved in these syndromes. The first Indonesian patient with Mowat-Wilson syndrome was molecularly diagnosed (**Chapter 5.1**). In a larger subgroup of individuals with microcephaly, however, no pathogenic mutations could be identified in the genes responsible for this disorder (**Chapter 5.2**).

Molecular screening on PW/AS could not identify genetic abnormalities in a subgroup of individuals with clinical suspicion of these syndromes in our population. The low frequency of PW/AS which has been reported to be 0.2–0.4 % in the ID population might be a plausible explanation why individuals with PW/AS could not be identified in this study (Stevenson et al., 2003; Rauch et al., 2006). Negative findings in the series of molecular analyses performed in subgroups of individuals with microcephaly/macrocephaly and epilepsy might be due to several factors, either genetic or non-genetic. First, there are several non-genetic factors which have been attributed to play a role in epilepsy, microcephaly and macrocephaly (Ivanovic et al., 2004; Tarrant et al., 2009; Ono and Galanopoulou, 2012). Second, the negative findings in the coding regions of the investigated genes indicate that mutations in these genes have a very low frequency, at least in our population. Third, it is suggested that genetic factors other than mutations in these genes might be involved in the aetiology of these conditions. Finally, another point of consideration is that we cannot rule out the presence of deep intronic mutations, promoter mutations, deletions or duplications in these genes, because these have not been investigated.

The total yield of aetiological diagnoses in this study was 20.5% (108/527), leaving 79.5% of individuals without a diagnosis. This percentage is comparable with the 21.8% (124/570) genetic diagnoses reported by Rauch et al., (2006) (excluding the molecular karyotyping result). Compared with the study conducted by Stevenson et al. (2003) in larger cohort of ID individuals, the number of diagnosis in our study is significantly lower ($p < 0.005$). In their study, in more than 40% of cases a diagnosis could be assigned. This difference, however, can easily be explained by the fact that also non-genetic factors such as environmental insults, infection, exposition to chemical substances and prematurity were evaluated. Moreover, some factors such as multifactorial and culturofamilial were determined. In addition, metabolic studies and neuroimaging studies were conducted in their study as well. The evaluation of non-

genetic factors, and metabolic and neuroimaging studies, however, were not conducted in our population. If metabolic studies and neuroimaging investigations had been performed in our population, this could have added an approximate 3.7%–4.6% of aetiological diagnosis (Michelson et al., 2011).

The use of state-of-the-art genetic analyses such as microarray analysis and NGS might increase the number of diagnosis in ID Individuals even further. It is estimated that microarray analysis adds approximately 12% of aetiological diagnoses (Miller et al., 2010; Cooper et al., 2011). Furthermore, investigation using WES would tally up another 8-16% of aetiological diagnosis (Dixon-Salazar et al., 2012; de Ligt et al., 2012). So, the total yield of aetiological diagnoses would be in the order of 44-53%. Further investigations in future studies, therefore, are warranted.

6.1.2. Establishing a diagnosis: a challenge in Indonesia

Understanding the challenges of establishing a diagnosis for ID individuals in Indonesia requires comprehensive reflection on the complex relationship between government policies, public healthcare systems, socio-cultural factors, economics and education. Government health policies in Indonesia continue to focus on infectious diseases, while limited attention is given to chronic and non-communicable disorders such as neurodevelopmental disorders. Consequently, the services for such disorders are disproportionately underfunded.

Starting from the last five years, the Government of the Republic of Indonesia has been providing health and social security insurance for the poor. However, the insurance is limited to basic medical costs. Sophisticated examinations such as brain imaging and genetic analysis, unfortunately, are not yet covered by such insurance. In addition, these health facilities often have minimal equipment and are only available in big cities. In some areas of Indonesia, individuals with ID and their families are still being stigmatized (Komardjaja, 2005). Therefore, parents tend to hide them from community. This, together with the high cost of special education that is not covered by the government, is the reason why a large number of individuals with ID stay at home without proper education and training. Consequently, they remain unidentified as such. Neonatal screening has not been established yet as a health policy in Indonesia due to lack of facilities, funding and human resources. Therefore, the incidence of preventable causes of ID identified by newborn screening remains unknown. It should be noted, however, that metabolic disorders causing ID such as congenital hypothyroidism are not uncommon (Rustama et al., 2003). In the health sector, the lack of medical personnel's knowledge and awareness of genetics and the virtual absence of clinical geneticists within the country complicate further diagnosing ID individuals. In addition, genetic research and its funding are very inadequate.

6.2. Implication of genetic aetiological diagnosis of ID individuals

The identification of the aetiology of ID is of major importance for patients and their families. Establishing a diagnosis means giving clear answers for the frequently asked questions among parents about what their offspring “have”. In majority of cases, therefore, it allows answers to multiple questions about causation and pathogenesis, recurrence risk, and early application of therapeutic and educational interventions (Baker et al., 2012). In addition, it aids family’s grieving and adaptation, as well as facilitates connections within groups/families with similar disorders (Lopez-Rangel et al., 2008).

Parents of children with ID of unknown aetiology will face one of the greatest challenges, i.e. searching for explanations “why it happened”. After the diagnosis of ID in their children, parents often showed initial reactions such as ambivalence, anger, confusion, self pity, blame, feelings of helplessness, depression, disappointment, grief, guilt, mourning, impulses to kill the child and suicidal impulses (Mary, 1990; Majumdar et al., 2005). The result of aetiological diagnosis sometimes helps to fade away these initial feelings. This will be followed by a reaction stage, in which they show emotion of denial, sadness, and anger. Gradually, parents will enter an adaptation stage where they begin to ask questions about what to do, and finally a reorganization stage where they seek help and begin to plan ahead (Mary, 1990; Majumdar et al., 2005). In this stage, again, aetiological diagnosis plays a role because it allows answers to parents’ multiple questions on management and prognosis. For medical practitioners, an aetiological diagnosis assists them to provide a general prognosis and treatment options and gives them the opportunity to counsel the individual/family regarding prospective health care needs and reproductive planning. In complicated patients, such as individuals with ID and MCA, it also allows a multidisciplinary medical management approach in which unnecessary, invasive and costly investigations and testing can be avoided. In addition, early prevention as well as early intervention for co-morbidities can be well planned (Lopez-Rangel et al., 2008). Caregivers and educational specialists may take benefits from the aetiological diagnosis. They can access additional resources for individuals with ID based on a specific diagnosis and known developmental and medical profile. In addition, they may be able to provide access to therapeutic recreational programmes, social programmes and group support for affected individuals as well (Baker et al., 2012).

There is no reason to believe that the above mentioned implications of establishing a diagnosis in individuals with ID are less important in Indonesia, compared to the rest of the world. By providing evidence of an important genetic contribution to the cause of ID, our study will strengthen the position of medical genetics in Indonesia and enhance genetic awareness of the Indonesian medical profession and the public at large. In addition, this hopefully will trigger the government to pay more attention to medical genetics, particularly on research funding and providing better facilities for genetic services in the public health system. To date,

genetics is still not incorporated in the curricula of Indonesian medical education. The results of our study will hopefully stimulate the Indonesian medical association and/or medical specialist collegiums to incorporate genetics into the medical education as well as into the training program for other health workers. The medical specialist collegiums play a significant role in determining curricula for training and education of each medical specialist.

6.3. Diagnostic protocol for Indonesia

6.3.1. Clinical guideline for the Indonesian setting

As no clinical guideline exists for the evaluation of ID individuals in Indonesia, establishing one for local use was one of the aims of this study. At the initial stage of this study, the clinical guidelines of the RUNMC Genetic Department, The Netherlands have been adopted and utilized to evaluate ID individuals. These guidelines are in accordance with existing guidelines of the American College of Medical Genetics and systematic studies of (Curry et al., 1997; van Karnebeek et al., 2005). Adaptations and improvements, however, need to be considered to adjust for local use. Mainly, this clinical guideline is intended to be utilized for routine use by paediatricians, neurologists and medical geneticists in evaluating individuals with ID. However, it can also be utilized for research purposes and by other medical workers.

The first step is exploring the essential elements of the affected individual's history that include a three-generation pedigree, and pre-, peri-, and postnatal history. A three-generation pedigree should be carefully taken. The presence of relevant clinical characteristics should be noted in family members, such as abnormal mental development and/or MCA (important clues for aetiology), psychiatric disorders, recurrent miscarriages (hint for chromosomal translocation), increased age of affected individual's parent (suspicion to spontaneous mutations), consanguinity (sign for autosomal recessive disorders), head circumference and education of parents (indication for normal family intelligence level). Careful history taking will include the pre- and perinatal history and care, especially questions concerning possible teratogenic exposures (alcohol, cigarettes, pesticides, herbal and/or alternative medicines, medications and radiography), maternal diabetes or infectious diseases during pregnancy as well as the amount of amniotic fluid and child movements (Curry et al., 1997; van Karnebeek et al., 2005). The presence of more similarly affected individuals in the family may highlight a distinctive pattern of inheritance, and may provide clues for a particular genetic disease. For example, an X-linked pattern may indicate that the Fragile-X syndrome is running in the family.

The information on the perinatal period will include length, weight and OFC at birth, signs of asphyxia/APGAR score, feeding and sleeping patterns as well as hypotonia in early life. The information on the postnatal period will include further weight, height and OFC curves as well as the developmental milestones. Specific attention should be paid to loss of already gained skills. A history of trauma, injury,

medication as well as illness history in the postnatal period should be noted. Comorbidity with other cognitive disorders - more frequent in ID individuals - should be identified, as it can facilitate the diagnostic process by permitting classification of ID patients into specific subgroups (i.e. autism, ADHD, epilepsy) (Curry et al., 1997; van Karnebeek et al., 2005). In Indonesia, history taking is often challenging. The parent's education level contributes to the success of history taking. Prenatal care is still considered as an uncommon practice, particularly in suburban and rural areas. Therefore, the information about the prenatal period is sometimes difficult to obtain. Many parents do not have or often forget the basic birth records such as weight, length and OFC, as they often give birth in their own house without attendance of medical personnel. In many cases, parents do not pay attention to their children's developmental milestones. Socio-cultural factors also complicate history taking, for instance, parents often associate the condition of their children with myth or belief. Likewise, parents are not always available as they might, in order to avoid embarrassment, have entrusted their ID child to the grandparents or to other relatives without giving them relevant data.

The next important step is physical examination. It should start with general physical measurements such as weight, height, head circumference (OFC), outer canthal distance (OCD), inner canthal distance (ICD), inter pupillary distance (IPD), ear length, total hand length (THL), palm length (PL), and -in males- testicular volume (Curry et al., 1997; van Karnebeek et al., 2005). Some other measurements, such as arm span and sitting height, should be performed if there is a suspicion of a specific condition. Important landmarks for the measurements can be found in Hall et al., (2007). Measurement results for height and weight, can be plotted to the normal graph of measurements which are now available for the Indonesian population (Batubara et al., 2006). This normal graph for Indonesian children is based on a nationwide survey, in which data from approximately 35,000 Indonesian children were compared to the United States of America Centers for Disease Control and Prevention (CDC) growth charts by calculating their individual standard deviations out of the SD reference values for the American population. A mean difference of -1.47 SDS for boys and -1.43 SDS for girls was found. Since the measurements of Batubara et al. (2006) only include OFC data for infants below 1 year of age, OFC measurements in this study were plotted to the Nellhaus' head circumference chart (Nellhaus, 1968). The choice to use the Nellhaus' charts is based on their mixed racial population sample, their frequent use in clinical settings, and the age range covered by these charts (Miles, 2000). However, we had the impression that the OFC of Indonesian children was generally lower than the mean values in the Nellhaus' charts. Indeed, our pilot study conducted in Semarang, Central Java, indicated that the mean value of head circumference of Indonesian normal school children aged 7-12 years is smaller compared to the normal range of the Nellhaus charts. The boys' mean value ($n=128$) was between the 3rd and under 50th percentile, while for girls ($n=146$) it lied between the 10th and under 50th percentile (Mundhofir et al, unpublished observations). The other measurements such as ICD,

IPD, OCD, ear length, can be plotted to the normal measurement graph published by (Hall et al., 2007). However, it is possible that the anthropometrical data for the Indonesian population might differ from those of other populations, which is supported by the study on length and weight of Batubara et al. (2006) and our pilot study on OFC.

The following step is clinical examination that comprises general habitus and dysmorphological assessment of craniofacial, thorax, abdomen, back, genitalia, upper and lower extremities (van Karnebeek et al., 2005; Baker et al., 2012). In an effort to make the dysmorphological terminology reliable and reproducible, the clinician should refer to the standard nomenclature formulated by an international group of clinicians working in the field of dysmorphology (Allanson et al., 2009; Biesecker et al., 2009; Carey et al., 2009; Hall et al., 2009; Hunter et al., 2009; Hennekam et al., 2009). A general neurological investigation is important since it may be a clue to a specific neurological condition. For instance, spasticity is invariably found in individuals who have had perinatal asphyxia or cerebral palsy. Likewise, it is important to pay attention to the skin and its appendages as it may be a clue to specific conditions such as neurofibromatosis or tuberous sclerosis (Seibert et al., 2011). Examining both vision and hearing is essential as disturbances of one or both have major implications for management and are often part of a recognizable syndrome (Sullivan et al., 2011). Abnormal behaviour should be noted as it may give clues to the aetiology of the ID (Dai et al., 2012; van Balkom et al., 2012; Marschik et al., 2012). A tool for the description of behaviour by Shalev and Hall, (2004) can be used. Finally, standard clinical pictures should be taken including standard face, side face, general body profile, side body profile, hands and feet. Pictures of other specific structures are only indicated if there is a peculiar appearance or dysmorphism.

Although the DSM-5 proposal does not list IQ test score requirements in the formal diagnostic criteria, it continues to specify that standardized psychological testing must be included in the assessment of ID individuals (American Psychiatric Association, 2012). Therefore, the next step is confirming the existence and the determination of the severity of disability by formal IQ testing.

6.3.2. Genetic, metabolic, and neuroimaging guidelines

Based on the results of this study, for routine practice and for future empirical studies aiming at detecting the aetiological diagnosis in individuals with ID, the guidelines on genetic, metabolic, and neuroimaging can be formulated as follows:

- a. In each individual, standard cytogenetic testing including fragile site screening should be performed, regardless of the degree of ID and the presence of dysmorphic features (Shaffer, 2005). The main reason for this is that cytogenetic analysis has the highest yield in the Indonesian population (basis: this study). Moreover, cytogenetic analysis is available and considered to be affordable. The analysis can be skipped only if non-genetic causes of ID are evident through history taking and physical examination. For individuals with

clinical features of a known chromosomal abnormality syndrome such as Down syndrome, still cytogenetic analysis should be performed, because the actual cytogenetic diagnosis may affect the recurrence risk (Shaffer, 2005).

- b. Molecular studies for FXS should be performed if the cytogenetic analysis is normal, regardless of sex, degree of ID, and the presence of associated features of FXS (basis: this study). Although the chance to identify FXS is ~1-3%, the detection remains of paramount importance because of its X-linked inheritance and therefore pertinent consequences for families. In addition, the presence of clinical features is sometimes subtle, particularly in women. The analysis can be skipped only if clinical features are strongly pointing to a specific known syndrome. The PCR based CGG repeat *FMR1* gene analysis is available in Indonesia and considered to be affordable.
- c. Molecular studies for submicroscopic rearrangements should be considered in the future if cytogenetic analysis and FXS analysis are normal. The facilities to detect such aberrations are not yet available in Indonesia; however, in the near future, these will be available. Although setting up MLPA analysis for the detection of subtelomeric aberrations would lead to the detection of 3% of ID causes (this study), it might be a better option to invest money in high resolution genome wide approaches (like SNP array). These techniques will be able to detect submicroscopic deletions and duplications throughout the genome, including the subtelomeric regions.
- d. If sequencing facilities for a specific syndrome are available, sequencing of specific genes should be considered for individuals with clinical features strongly associated with that syndrome. As long as these facilities are not available in Indonesia, it is possible to send samples to diagnostic centres abroad in order to molecularly confirm the specific syndrome.
- e. If sophisticated genetic analyses such as whole exome sequencing will be available in the future, this analysis should be considered for individuals with negative results after extensive conventional genetic analyses (Baker et al., 2012; Dixon-Salazar et al., 2012; de Ligt et al., 2012). In the future, such state-of-the-art genetic analyses may replace those conventional tests.
- f. If facilities for metabolic investigations will be available in the future, these analyses can be considered for ID individuals. Some of metabolic disorders, although it has a low yield, are known to be treatable if diagnosed early in life (van Karnebeek, 2012); take for example, phenylketonuria and congenital hypothyroidism. Therefore, the screening for these disorders should be performed during neonatal period. The neonatal screening for such disorders should receive attention from the government of Republic Indonesia and should become one of the public health priorities in Indonesia.
- g. Neuroradiologic/brain imaging facilities are available but at a limited scale and expensive. Neurological imaging studies have a high yield for brain abnormalities, but low for establishing aetiological diagnoses (van Karnebeek,

2005). Therefore, such studies should not be performed in each individual, but only if specific symptoms are present such as abnormal head circumference, focal neurological findings and symptoms at clinical history taking or physical examination that indicate a high chance for a brain anomaly.

Points a and b are highly recommended in a routine setting. Points c, d, e, f and g are recommended for future studies aiming at detecting the aetiological diagnosis in individuals with ID. It should be taken into account that points c, d, e, f and g can also be considered for routine setting in the future, when these facilities are available and affordable in Indonesia.

6.3. 3. Flowchart of diagnostic protocol

Following is the proposed flowchart of the diagnostic protocol for Indonesian ID individuals (see Table 6.1). Although this flowchart is designed to be applied nation wide, it should be considered that at this moment in many areas mainly outside of Java Island are of minimum facilities thereby laboratory/additional investigations cannot be performed. This can be anticipated with referring the patient to the nearest facility. In addition, some additional investigations, however, are not yet available in Indonesia. Collaboration with other centres abroad is needed to overcome this problem.

Table 6.1. Proposed flowchart of the diagnostic protocol for Indonesian ID individuals

No.	Step	Remarks
1.	History taking Family history Three generation pedigree	Including: stillbirths, miscarriages; history of ID, psychiatric disorders and developmental delay/learning difficulty; consanguinity, increased age and education level of parents
	Medical history / developmental history	
	Prenatal	Including: possible teratogenic exposures, prenatal infections, maternal diabetes, amount of amniotic fluid and movement of the foetus
	Perinatal	Including: delivery process, birth measurements, feeding, sleeping pattern, hypotonia
	Postnatal	Including: growth parameters, developmental milestones, history of trauma/injury, illness and

		medication, surgery, seizures, regression.
2.	Clinical examination	
	Physical measurements	Including: length, weight, OFC, OCD, ICD, IPD, THL, PL, testicular volume (in male). On special indications: arm span, sitting height.
	General aspect and dysmorphological assessment	Including: general habitus, craniofacial, thorax, abdomen, genitalia, upper and lower extremities
	Other examination	Including: general neurological investigation, skin and appendages, hearing and vision.
	Behaviour	Including: general impression of the personality, peculiar manners: posture, movement, walking, mimic and stereotypes.
	Clinical photographs	Including: standard face, side face, general body profile, side body profile, hands and feet; other structures on indication.
3.	IQ data	If no IQ data available, refer the individual to psychologist. Retest if IQ data is different from actual observation or testing was not done recently.
4.	Laboratory/technical investigation	
	Chromosome analysis	For each individual: including standard GTG banding and other banding on indication.
	<i>FMR-1</i> PCR analysis	For each individual with normal chromosome analysis.
	Submicroscopic rearrangements	For each individual with normal chromosome analysis and <i>FMR-1</i> PCR analysis and/or suspicion of CNV/microdeletion syndrome
5.	Additional investigation on indication	
	Investigation on specific monogenic diseases	Suspicion of other monogenic diseases
	WGS/WES	When the conventional genetic analyses could not identify the cause

Neuroimaging	of ID and genetic abnormality has been strongly suspected. Neurological problems, macrocephaly, microcephaly, cranial contour abnormalities, epilepsy, focal neurological findings
Metabolic	Suspicion of metabolic diseases

6.4. Recommendations and future directions

6.4.1. Recommendations

Diagnostic evaluation of an ID individual ideally should be performed with a minimal burden for the affected individuals, parents and society. In addition, it should be simple, cheap and minimally invasive. On the other hand, the evaluation should be evidence based and using the newest developments for which it may need sophisticated and expensive technology (Baker et al., 2012). Protocols and guidelines for diagnostic evaluation should be arranged such as to be both up-to-date and feasible (Sullivan et al., 2011). Nevertheless, local setting, availability of diagnostic facilities and financial aspects should be taken into account. Based on the results of this thesis the following is recommended for the Indonesian setting:

1. As an aetiological diagnostic investigation is of utmost importance for ID individuals, parents, medical practitioners and care-givers, a nation-wide study is recommended for ID individuals with at least chromosome analysis and *FMR1* analysis. Therefore, the government of Republic Indonesia and private initiatives should allocate a budget in the health insurance for such a study. Furthermore, they should allocate budget to develop diagnostic facilities and human resources for genetic diseases.
2. This study proposes a protocol and guidelines for the diagnostic investigation of ID individuals. One of the essential steps is history taking, which is often very difficult to obtain due to factors described elsewhere in the thesis (chapter 6.1.2). Furthermore, perinatal data is of the utmost importance in determining the aetiology of ID. Acquired disorders during pregnancy, delivery, or the neonatal period are important causes of ID. Further improvement of perinatal care and registration of basic data on pregnancy, delivery and neonatal period in every newborn are needed. In order to avoid unnecessary expensive laboratory investigations in individuals with acquired disorders, the availability of such information is needed. It is recommended, therefore, that

such information be obtained from parents and/or health care facilities prior to admission of ID individuals to schools and institutions. In addition, schools and institution should keep these data on affected individuals. This is eventually to reduce costs and to increase the yield of expensive genetic analysis.

3. Medical students, residents and specialists such as paediatricians or neurologists should have a proper training in the field of genetics, dysmorphology and syndromes in general. Furthermore, these clinicians should have access to the major dysmorphology textbooks, electronic catalogues (e.g. London Dysmorphology Database and Possum) as well as online databases (e.g. Phenomizer and ECARUCA). It should be taken into account that routine laboratory analysis is less cost effective than targeted diagnostic work-up which is preceded by a clinical diagnosis of a specific syndrome. In addition, properly trained physicians will be in a far better position to explain to parents about the results of cytogenetic and molecular analyses.
4. In effort to provide a better service to ID individuals in the context of genetic and acquired disorders, basic need of genetics department should be fulfilled. First, properly trained physician in genetic, dysmorphology and acquired disorders should be available. Second, basic clinic for clinical genetics should be present. Finally, basic laboratory for cytogenetic, molecular and metabolic disorders should be available. It should be considered that, the cost invested in the basic need of this department is much lower than the investment in sophisticated apparatus such as for DNA microarray and WGS/WES. In addition, fulfilling basic need of genetic department is more cost effective to be implemented in Indonesian setting. Therefore, providing basic need for genetics department should be one of Indonesian public health priorities.
5. Common questions of parents of affected individuals are related to management and treatment. Since for most genetic diseases, there is no cure, priority should be given to the treatment of co-morbidities as well as behavioural disorders and wherever possible their prevention. In order to maintain continuity of care, a complete record of all medical interventions, and multidisciplinary management is important. It is also recommended that multidisciplinary teams consisting

of psychologists, medical specialists/medical practitioners, therapists and caregivers should be established in every centre of care, such as schools and institutions.

6. Recurrences are the concern of the affected individual's parents as well as healthy siblings. These concerns must be met with genetic counselling. In Indonesia, however, this is an uncommon practice. It is recommended that the government provides genetic counselling services at least in tertiary health care facilities and furthermore recognizes a genetic counsellor as an official or registered professional. Nowadays, the University of Diponegoro has an official training at master level for genetic counsellor. However, it is still not officially recognized throughout the country.
7. Many individuals with ID have complex health issues, which are different from those of the general population. Primary care for individuals with ID allows early detection and intervention of particular health issues faced by them. These are important in an effort to improve their quality of life, to improve their access to health care, and to prevent suffering, morbidity and premature death. Therefore, the establishment of primary care providers specialized for ID individuals should be considered as a health priority.
8. Education is one of the most elementary rights of every citizen. The law in Indonesia validates that every citizen, including people with intellectual disability, has equal rights and opportunities to get quality education. However, the accessibility of individuals with ID to facilities, which would allow them to enjoy equal opportunities to participate in education, is still limited. Nowadays, their non-ID counterparts take the benefit from free education policy; however, this is not the case for ID individuals where only few institutions and schools are free of charge (available only in the big cities). The Indonesian Government as well as public-private partnerships are encouraged to provide better education facilities for them. Furthermore, free education for ID individuals should be funded from the national budget.
9. Parent support groups for ID individuals, which now exist only in the capital and other big cities, have to be initiated nationwide.

6.4.2. Future directions

Research

1. In the present study, we mainly focused on the genetic aetiological diagnostic process. However, financial constraints hampered the use of state-of-the-art technologies such as array analysis and WES. The fact that we identified only about 20% of genetic abnormalities in the population of Indonesian ID leaves sufficient room for future studies in our cohort using the above mentioned technologies.
2. In effort to reduce research cost, the availability of reliable perinatal data and properly trained doctors in genetics and acquired disorders should be considered for future studies. In addition, intense screening for perinatal and acquired disorders should be performed prior to genetic research.
3. Normal measurement graphs for the Indonesian population are limited to height and weight, and OFC for infants; therefore, further studies of other physical measurements should be conducted nationwide.
4. Protocols and guidelines for diagnostic evaluation of Indonesian ID individuals which are produced from this thesis may need to be evaluated in the future. Studies about the validity and yield of the screening recommendations are needed.
5. In the present study, we performed genetic counselling when a genetic diagnosis was obtained. Studying the psychological effects of these counselling sessions in the family are needed in the future.

Care

1. Nowadays, the accurate number of Indonesian ID Individuals is not known (**chapter 1.1.4**). Knowing the accurate number of ID individuals is of paramount importance in effort to plan research, care and management. A nationwide coordination centre can be supportive in registering individuals with ID. This centre may be helpful in offering easy access for government, researchers, care providers and general practitioners. In addition, it may have a task in the education of general practitioners and care providers. This centre also may provide further research.
2. Comprehensive clinics for ID individuals are of major importance for evaluating ID individuals. However, there are only 2 such

comprehensive clinics in Indonesia, which is very inadequate. In addition, facilities and human resources are far from ideal. The establishment of new comprehensive clinics for ID should be considered, at least one in each major city. When the diagnosis of known syndrome has been established, the presence of such clinics is vital to monitor its known comorbidities. This eventually will help the affected individuals to receive a better management.

3. Certified clinical geneticist training and the establishment of a human genetics association in the country should be initiated.
4. Improvement of perinatal care and registration of basic data on pregnancy, delivery and neonatal period in every newborn are critically important. Health care facilities at all levels, such as health centres, hospitals, maternity and paediatric clinics should improve their ability in these fields.
5. The implementation of medical genetics in the Indonesian public health system is still facing some complicated issues. Nowadays, establishing an aetiological diagnosis and providing possibilities for carrier testing are still not considered to be useful. This is due to the lack of awareness of medical personnel and the public at large. Therefore, public dissemination and education through workshops and seminars about genetics are of paramount importance.

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Summary / Samenvatting / Intisari

Acknowledgments

List of Publications

Curriculum Vitae

Appendix

Summary

Intellectual disability (ID) has been defined as a significant limitation in both intellectual functioning ($IQ < 70$) and concurrent limitations in conceptual, social and practical adaptive skills, manifesting before the age of 18. The aetiology of ID is complex and heterogeneous and includes genetic, non-genetic (environmental) and multifactorial causes. The details about known genetic aetiologies associated with ID are discussed in **chapter 1**.

Indonesia ranks fourth on the list of countries with the largest population in the world, after China, India, and the United States, indicating that also the ID population must be large. However, only a partial genetic diagnostic work-up of the ID population in Indonesia has been performed. This study aimed at recording an aetiological diagnosis of ID as well as trying to determine the prevalence of cytogenetic and some molecular diagnoses, performing phenotype-genotype correlation studies, and defining a diagnostic protocol for ID which is applicable in the Indonesian context.

In **chapter 2** we determined the frequency of cytogenetic abnormalities in a cohort of Indonesian individuals with ID using conventional cytogenetic analysis confirmed by some stand-alone molecular tests. Chromosomal abnormalities were found in 87 (16.5%) out of the 527 intellectually disabled individuals, with trisomy 21 being the major chromosomal abnormality, occurring in 74 cases (14%). The detection rate of our study is in the range of the latest meta-analysis study conducted in ID individuals.

In **chapter 3** we determined the frequency of Fragile X syndrome (FXS) in males and females, and documented their relatives with a similar disorder. Cytogenetic analyses revealed that five individuals (four males and one female) out of 87 had FXS. Since FXS cannot always be identified by cytogenetic analysis, a follow up study using molecular analyses was conducted in the 440 samples (279 males and 161 females) without chromosomal abnormalities. This resulted in the identification of four additional cases (three females and one male). In the Indonesian ID population, the prevalence of FXS is therefore, 9/527 (1.7%), while the prevalence of FXS in males and females is 1.5% (5/329) and 2% (4/198), respectively. The detection rate of FXS in our study is comparable with the latest meta-analysis study.

In **chapter 4** we determined the frequency of subtelomeric deletions and duplications using MLPA in a cohort of Indonesian individuals with ID who had a normal karyotype and who were FXS negative. The frequency of subtelomeric rearrangements in this study was 3.7% (16/436). In addition, in one of the positive individuals, we identified a monosomy 9pter and trisomy 9q34.11qter, which appeared also to be present in her sister. The familial nature of this rearrangement is explained by a

maternal pericentric inversion. Our results on subtelomeric rearrangements are comparable with the latest meta-analysis study.

In **chapter 5** we investigated a subset of ID individuals suspected of having a specific syndrome. A case with Mowat-Wilson syndrome and the results of sequencing of several genes involved in autosomal recessive primary microcephaly are described.

In **chapter 6**, the implication of this work including the establishment of a diagnostic protocol for the Indonesian setting is described. In addition, a general discussion and future directions towards a better genetic diagnostic workup in Indonesia having limited laboratory facilities are also provided.

In summary, this thesis provides evidence that clinical evaluation and relevant genetic investigations can achieve diagnoses in a significant proportion of cases. In spite of the fact that the diagnostic possibilities in developed countries are far more advanced than the tests discussed in this thesis, it is evident that our studies are a fundamental step for aetiological diagnostic evaluation of ID individuals in Indonesia. As copy number variations, single nucleotide variations, and metabolic disorders are causes of ID that were not studied in our cohort, these causes call for further investigation in future studies.

Samenvatting

Intellectual disability (ID), in het Nederlands verstandelijke beperking, wordt gedefinieerd als een significante beperking in zowel intellectueel functioneren ($IQ < 70$) als beperkingen in conceptuele, sociale en praktische adaptieve vaardigheden, die vóór het 18^{de} levensjaar tot uiting komen. De etiologie van ID is complex en omvat genetische en niet-genetische factoren (omgeving) of een combinatie hiervan. De details over de bekende genetische oorzaken geassocieerd met ID worden besproken in **hoofdstuk 1**.

Indonesië heeft na China, India en de Verenigde Staten, de grootste bevolking ter wereld, wat suggereert dat ook de ID populatie groot moet zijn. Echter, de genetische studies die zijn uitgevoerd bij de ID bevolking van Indonesië zijn zeer beperkt. Deze studie is gericht op het stellen van etiologische diagnoses voor ID waarbij de prevalentie van cytogenetische en een aantal moleculaire diagnoses in kaart wordt gebracht, op het uitvoeren van fenotype-genotype correlatie studies en op het definiëren van een diagnostisch protocol voor ID in de Indonesische context.

In **hoofdstuk 2** hebben we de frequentie van cytogenetische afwijkingen in een cohort van Indonesische individuen met ID bepaald met behulp van conventionele cytogenetische analyses, die bevestigd zijn met een onafhankelijke moleculaire test. Bij 87 van de 527 verstandelijk beperkte individuen (16,5%) werd een chromosomale afwijking gevonden, waarbij trisomie 21 de meest voorkomende chromosoom afwijking was, gevonden bij 74 individuen (14%). De detectie ratio in onze studie ligt binnen de grenzen van de meest recente meta-analyse studies uitgevoerd bij ID individuen.

In **hoofdstuk 3** hebben we de frequentie van het Fragiele X syndroom (FXS) bepaald bij mannen en vrouwen, en zijn hun aangedane familieleden gedocumenteerd. Vijf personen (vier mannen en een vrouw) van de 87 met een cytogenetische afwijking hadden FXS. Omdat FXS niet altijd met cytogenetische testen kan worden aangetoond, werd een vervolgstudie met moleculaire analyses uitgevoerd bij de 440 individuen (279 mannen en 161 vrouwen) zonder chromosomale afwijking. Dit resulteerde in de identificatie van vier extra gevallen (één man en drie vrouwen) met FXS. In de Indonesische ID populatie is de prevalentie van FXS dus 9/527 (1,7%), waarbij de prevalentie bij mannen en vrouwen respectievelijk 1,5% (5/329) en 2% (4/198) is. De detectie ratio van FXS in onze studie is vergelijkbaar met die in recente meta-analyse studies.

In **hoofdstuk 4** hebben we met MLPA de frequentie van subtelomere deleties en duplicaties bepaald in een cohort van Indonesische individuen met ID, die een normaal karyotype hadden en FXS negatief waren. De frequentie van subtelomere afwijkingen in deze studie was 3,7% (16/436). In een van de positieve individuen hebben we een monosomie 9pter en een trisomie 9q34.11qter geïdentificeerd, die ook aanwezig bleek te zijn bij haar zus. De aanwezigheid van deze chromosomale afwijking bij twee kinderen kon verklaard worden door een pericentrische inversie bij de moeder. Onze resultaten m.b.t. subtelomere afwijkingen zijn vergelijkbaar met de meest recente meta-analyse studie.

In **hoofdstuk 5** hebben we een gedeelte van de ID individuen, die er van verdacht werden een specifiek syndroom te hebben, onderzocht. Een casus met Mowat-Wilson syndroom en de resultaten van de analyse van genen betrokken bij microcefalie worden beschreven.

In **hoofdstuk 6** worden de implicaties van het onderzoek besproken, inclusief het opstellen van een diagnostisch protocol voor Indonesisch gebruik. Daarnaast wordt een algemene discussie en toekomst perspectief op een betere genetische diagnostiek in Indonesië gegeven.

Samengevat, levert dit proefschrift het bewijs dat een klinische evaluatie en relevant genetisch onderzoek kan leiden tot een diagnose in een significant deel van de gevallen. Ondanks het feit dat de diagnostische mogelijkheden in ontwikkelde landen veel groter zijn dan de testen die in dit proefschrift worden besproken, is het duidelijk dat onze studies een belangrijke bijdrage leveren aan de ontwikkeling van een etiologische diagnostische evaluatie van ID individuen in Indonesië. Omdat geen onderzoek is gedaan naar veranderingen in kopie nummer, naar veranderingen in de sequentie, en naar metabole aandoeningen als oorzaak van ID, ligt er nog een weg open voor toekomstig onderzoek.

Intisari

Disabilitas intelektual (DI) didefinisikan sebagai keterbatasan fungsi intelektual dibawah rata-rata ($IQ < 70$) yang disertai dengan keterbatasan dalam melakukan aktivitas sehari-hari, berfikir secara konseptual dan kritis, serta dalam menyesuaikan diri dengan masalah dan kondisi situasi sosial. Keterbatasan-keterbatasan ini terjadi sebelum usia 18 tahun. DI mempunyai etiologi yang kompleks dan heterogen, termasuk didalamnya penyebab genetik, non-genetik (lingkungan) dan multifaktorial. Etiologi genetik yang terkait dengan DI dibahas dalam **bab 1**.

Indonesia menempati urutan keempat negara-negara dengan populasi terbesar di dunia, setelah China, India, dan Amerika Serikat, menandakan jumlah penduduk dengan DI juga banyak. Namun begitu, diagnosis genetik yang telah dilakukan selama ini pada populasi DI di Indonesia hanya sedikit. Penelitian ini bertujuan untuk mendata diagnosis etiologi dari DI, menentukan prevalensi kelainan kromosom dan beberapa kelainan molekuler, melakukan studi korelasi fenotip genotip, dan menegakkan protokol diagnostik untuk DI yang sesuai dengan kondisi di Indonesia.

Dalam **bab 2** dibahas tentang frekuensi kelainan kromosom pada individu dengan DI di Indonesia menggunakan analisis kromosom konvensional yang dikonfirmasi dengan beberapa tes molekuler independen. Kelainan kromosom ditemukan pada 87 (16,5%) dari 527 individu dengan DI dimana trisomi 21 menjadi kelainan kromosom utama yang terjadi pada 74 kasus (14%). Penelitian ini sesuai dengan studi meta-analisis terbaru yang dilakukan pada individu dengan DI yang telah dilakukan sebelumnya.

Dalam **bab 3** dikemukakan tentang frekuensi *Fragile X syndrome* (FXS) pada laki-laki dan perempuan yang selanjutnya dilakukan pendataan pada kerabat yang mempunyai gangguan yang sama. Analisis kromosom mengungkapkan bahwa lima orang (empat laki-laki dan satu perempuan) dari 87 memiliki FXS. Karena FXS tidak selalu dapat diidentifikasi dengan analisis kromosom, maka penelitian lanjutan menggunakan analisis molekuler dilakukan pada 440 sampel (279 laki-laki dan 161 perempuan) yang tidak mempunyai kelainan kromosom. Studi lanjutan ini mengidentifikasi empat kasus tambahan (tiga perempuan dan satu laki-laki), sehingga pada populasi DI di Indonesia prevalensi FXS adalah 9/527 (1,7%), dimana prevalensi FXS pada masing-masing laki-laki dan perempuan adalah 1,5% (5/329) dan 2% (4/198). Tingkat deteksi FXS dalam penelitian ini sebanding dengan studi meta-analisis terbaru.

Dalam **bab 4** frekuensi kelainan delesi dan duplikasi subtelomerik telah diidentifikasi menggunakan metode *Multiplex Ligation-dependent Probe Amplification*

pada individu dengan DI di Indonesia yang memiliki kariotipe normal dan FXS negatif. Frekuensi kelainan tersebut dalam penelitian ini adalah 3,7% (16/436). Selain itu, pada salah satu individu dengan kelainan ini, kami mengidentifikasi kelainan 9pter monosomi dan trisomi 9q34.11qter yang terjadi juga pada adik perempuannya. Kelainan ini terjadi akibat adanya inversi perisentrik pada ibunya. Hasil studi tentang delesi dan duplikasi subtelomerik sebanding dengan studi meta-analisis terbaru.

Dalam **bab 5** dibahas tentang penelitian pada sebagian dari individu dengan DI yang diduga memiliki sindrom tertentu. Pada bab tersebut ditampilkan sebuah kasus *Mowat-Wilson syndrome* dan hasil penelitian menggunakan metode sekuensing pada gen-gen yang terlibat dalam autosomal resesif microcephaly primer.

Dalam **bab 6**, dibahas secara umum implikasi yang ditimbulkan dari penelitian ini termasuk didalamnya dikemukakan penegakan protokol diagnostik yang dapat digunakan di Indonesia. Selain itu disampaikan saran-saran demi terciptanya pemeriksaan diagnostik genetik yang lebih baik di Indonesia dengan adanya keterbatasan fasilitas laboratorium.

Sebagai intisari, tesis ini memberikan bukti bahwa evaluasi klinis dan pemeriksaan genetik yang relevan dapat menegakkan diagnosis yang signifikan pada individu dengan DI. Terlepas dari kenyataan bahwa diagnostik genetik di negara maju jauh lebih berkembang, namun demikian hasil penelitian ini menyajikan langkah mendasar untuk evaluasi etiologi diagnostik pada individu dengan DI di Indonesia. Beberapa penyebab DI tidak semuanya dapat diselidiki dalam penelitian ini antara lain *copy number variations*, *single nucleotide variations* dan gangguan metabolisme. Oleh karena itu, penelitian ini perlu dilanjutkan di masa datang.

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I wish to thank Clive Michello, Riski Lestiono and Alsya Affandy for proof reading my manuscripts. I thank the people from Department of Human Genetic RUNMC for their help and friendly relationship they have shared over time. There are too many to mention by name, but in particular drs. Mariken Ruiter, dr. Ineke van der Burgt, drs. Ilse Feenstra, drs. Carlo Marcelis, dr. Tjitske Kleefstra, dr. Marjolein Willemsen, dr. Tony Roscioli, Prof. Frans Cremers, Edward van Teeuwen, Menno Tjon-Pon-Fong, Hicham Ouchene, Rowdy Meijer, Alejandro EstradaCuzcano, Bjorn Bakker, dr. Daniella Kasteel, Galuh Dyah Nurastuti and Prof. dr. Hans van Bokhoven.

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1. **Mundhofir FEP**, Kooper AJ, Winarni TI, Smits AP, Faradz SM, Hamel BC (2010). A small (sSMC) chromosome 22 due to a maternal translocation between chromosomes 8 and 22: a case report. *Genet Couns* 21:99-108
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3. Winarni TI, Utari A, **Mundhofir FEP**, Tong T, Durbin-Johnson B, Faradz SM, Tassone F (2012). Identification of expanded alleles of the *FMR1* gene among high-risk population in Indonesia by using blood spot screening. *Genet Test Mol Biomarkers* 16:162-166
4. **Mundhofir FEP**, Winarni TI, van Bon BW, Aminah S, Nillesen WM, Merckx G, Smeets D, Hamel BC, Faradz SM, Yntema HG (2012). A cytogenetic study in a large population of intellectually disabled Indonesians. *Genet Test Mol Biomarkers* 16:412-7
5. **Mundhofir FEP**, Winarni TI, Nillesen W, van Bon BWM, Schepens M, Ruiterkamp-Versteeg M, Hamel BCJ, Yntema HG, Faradz SMH (2012). Prevalence of Fragile X syndrome in males and females in Indonesia. *World J Med Genet* 2:15-22
6. **Mundhofir FEP**, Smeets D, Nillesen W, Winarni TI, Yntema HG, de Leeuw N, Hamel BCJ, Faradz SMH, van Bon BWM (2012). Monosomy 9pter and trisomy 9q34.11qter in two sisters due to a maternal pericentric inversion. *Gene* 511(2):451-4
7. Winarni TI, **Mundhofir FEP**, Ediati A, Belladonna M, Nillesen WM, Yntema HG, Hamel BC, Faradz SM, Hagerman RJ (2012). The fragile X-associated tremor ataxia syndrome (FXTAS) in Indonesia. *Clin Genet* doi: 10.1111/j.1399-0004.2012.01899.x. [Epub ahead of print]
8. **Mundhofir FEP**, Yntema HG, van der Burgt I, Hamel BCJ, Faradz SMH, van Bon BWM. Mowat–Wilson syndrome: The first clinical and molecular report of an Indonesian patient. *Case Rep Genet*. Article ID 949507, doi:10.1155/2012/949507
9. **Mundhofir FEP**, van Bon BWM, Winarni TI, Sistermans EA, Faradz SMH, Hamel BCJ (2012). p.Ser252Trp and p.Pro253Arg mutations in *FGFR2* causing Apert Syndrome: The first clinical and molecular report of Indonesian patients. [Accepted: *Singapore Med J*]
10. Winarni TI, Utari A, **Mundhofir FEP**, Hagerman RJ, Faradz SMH (2013). Fragile X syndrome: Clinical, cytogenetics and molecular screening among autism spectrum disorder children in Indonesia. [Accepted: *Clin Genet*]

Curriculum vitae

Farmaditya EP Mundhofir was born on 25th of April 1981 in Jepara, Central Java, Indonesia. He finished his high school in Kudus in the year of 1998. In the same year, he continued his education at the Faculty of Social and Political Science majoring in Mass Communication in UNS Solo. In 1999, he moved to the Faculty of Medicine Diponegoro University (FMDU) Semarang and received his medical doctor degree in 2006. During his study at the Medical Faculty, he had been granted awards from FMDU as an Outstanding Student in 2001 and 2002. He received the Exxon-Mobil Oil scholarship for period of 2002-2004 as well.



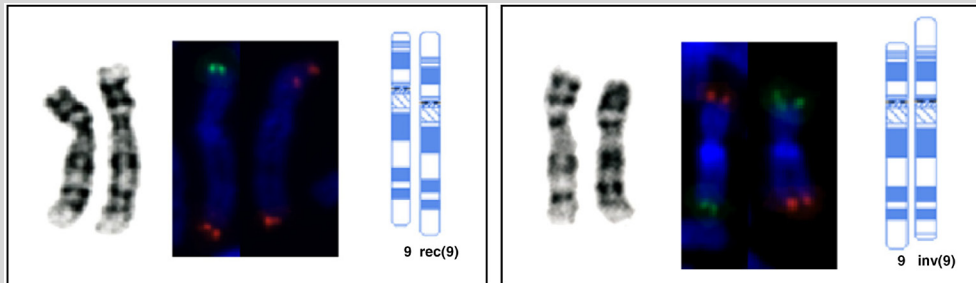
Shortly after his graduation, he started working at the Molecular and Cytogenetic Unit of Center for Biomedical Research and Department of Histology at the same university. He received a 2-years-research grant from the Ministry of Health in 2007. In the same year, he started his postgraduate education in the Master Program of Biomedical Science majoring in Genetic Counseling at the Diponegoro University with the scholarship (Excellent Scholarship/*Beasiswa Unggulan*) from the Ministry of National Education of the Republic of Indonesia. For his M.Sc research, he performed an internship in the Department of Human Genetics at the Radboud University Nijmegen Medical Centre (RUNMC), Nijmegen, The Netherlands for a year. During his M.Sc program, he received a fellowship to join a course of Medical Genetics held by the European Genetic Foundation in Bertinoro, Italy in which he was awarded a Best Poster Award. He received his M.Sc in 2009 with honor/*cum laude*. He received a post-graduate scholarship from the Ministry of National Education Republic of Indonesia for his PhD education. His PhD research is also supported by a Radboud University Fellowship. His current research is genetics of intellectual disability in Indonesian population (PhD thesis supervisors: Prof. dr. B.C.J Hamel, Prof. dr. S.M.H. Faradz and Dr. H.G. IJntema). The results of his PhD project are described in this thesis.

Some of his research was presented in national and international conferences. He received best poster in the annual national meeting of Indonesian Anatomist in Semarang on 2007. He presented his research on the International Workshop on Mental Retardation in Venice (2007) and in Berlin (2011), on the Human Genome Meeting (HGM) in Dubai (2011) and in Sydney (2012), and on and Asia Pacific Conference of Human Genetics in Kuala Lumpur (2012). He is a member of Human Genome Organization (HUGO) and Asia Pacific Society of Human Genetics (APSHG). He is married to Riena Anggraeni and lives in Semarang, Central Java, Indonesia.

Appendix (Color Figures)

Chapter 4.2, figure 1

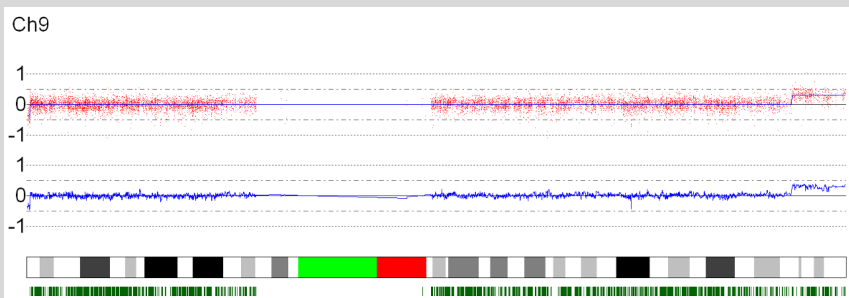
Figure 1. Conventional cytogenetic analysis, FISH analysis and the ideogram of the aberration in the patient (left) and her mother (right).



Chromosome 9 of the patient showed a duplication of 9q material attached to the p-arm of the chromosome (left). FISH results with chromosome 9p telomere-specific probe (green) and chromosome 9q telomere-specific probe (red); Note the two signals of 9q on the patient's aberrant chromosome 9.

Chapter 4.2, figure 2

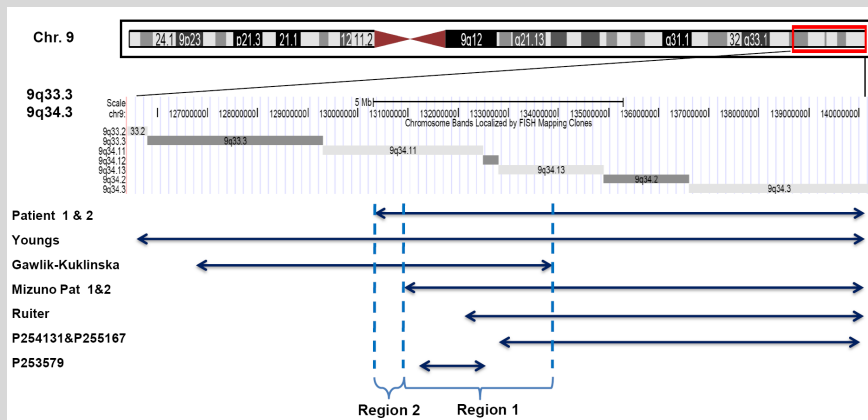
Figure 2. 250k SNP array result



A 9pter loss of 460 kb (containing 55 SNPs, 4 genes) and a 8.9 Mb gain of the 9q34.11q34.3 region (containing 394 SNPs, 162 genes) were observed.

Chapter 4.2, figure 3

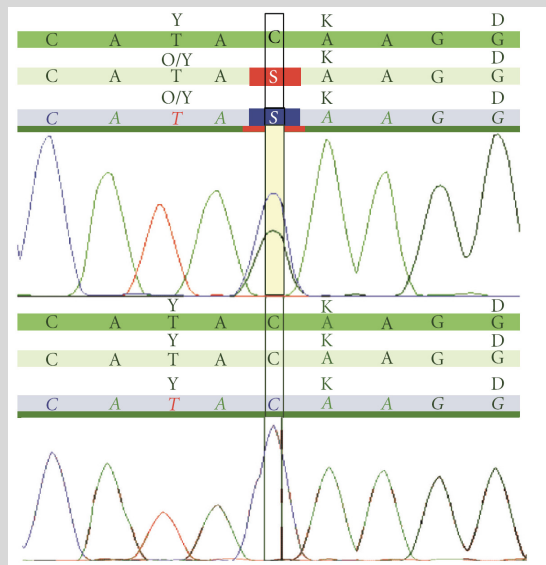
Figure 3. Critical regions associated with duplication 9qter.



This schematic illustration is based on hg18. All molecularly well characterized 9qter duplications are depicted by arrows. Region 1 has been associated with ID, strabismus, hypotonia, micrognathia, microstomia, arachnodactyly and sandal gap. The second critical region (Region 2) is associated with facial asymmetry and abnormal behaviour.

Chapter 5.1, figure 2

Figure 2: Electropherogram of molecular analysis in the patient sample.



The upper panel shows the heterozygous c.1965C>G (p.Tyr652X) mutation and the lower panel shows the wild type (control). The "S" on the electropherogram represents the C/G heterozygote.

